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RETINAL DEVELOPMENT DURING THE THIRD
LARVAL INSTAR OF DROSOPHILA MELANOGASTER

Thesis presented for the Degree
of Doctor of Philosophy of the
Open University in the Faculty
of Science

by

ANDREW TOMLINSON, B.Sc. (Manchester)

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SUMMARY

One of the major aims of the developmental biologist is to establish how a highly complex multicellular organism is derived from its unicellular zygotic origin. This thesis describes an investigation into the retinal development of Drosophila melanogaster during its third larval instar in an attempt to understand how the highly ordered and complex cellular constitution of the retina is established from a group of unorganised and undifferentiated cells.

The text is divided into six chapters. The first chapter is a review of the literature germane to the problem being investigated, and the second deals with the experimental methodology adopted. The remaining chapters relate the experimental findings and their implications to the problem under investigation.

Chapter Three describes the main body of the research, which consists of an electron microscopic serial section investigation of the developing retinal tissue, and the utilisation of computer graphic reconstruction techniques to give a comprehensive understanding of the three dimensional cellular arrangements.

The fourth chapter deals with autoradiographical studies which were undertaken to resolve an incongruity between a finding from the electron microscopic studies and an earlier literature report.

Chapter Five relates an investigation into the mutation sevenless which was performed in an attempt to both assess the value of knowledge acquired from the earlier investigations, and to understand the developmental failure that leads to this particular mutant phenotype.

The last chapter is a brief summary of how the retinal development during the third larval instar is better understood after the investigations described in this thesis.

CHAPTER 1. AN INTRODUCTORY REVIEW OF THE LITERATURE

1. A General Description of the Adult Eye

The eyes of Drosophila melanogaster are typically dull red, hemispherical in shape, and located on either side of the head. They are large in relation to the other head structures and thus appear as the dominant aspect of the head. The eye is compound by construction, consisting of approximately 700 - 800 sub-units which collectively act as the visual organ. Each individual sub unit - the so termed ommatidium, bears at its exterior a transparent, chitinous, corneal covering which is secreted by the underlying cone cells. Each individual cornea, or facet, is surrounded by six other such facets producing an extremely orderly hexagonal array. The centre of the facet protrudes from the eye to a greater extent than the edges, producing a cap for each ommatidium. Each facet, situated within its hexagonal array, bears at every other vertex a bristle of hair, functioning as a tactile organ Perry (1968b) (Fig. 1.1). Proximal to each cornea lies a group of twenty cells which collectively comprise the ommatidium (Ready et al, 1976). Eight of these cells are the photoreceptor cells, forming the functional light sensitive apparatus, four are corneal secreting - the cone cells, six are pigment cells of three distinct types, and the remaining two cells make up the bristle tactile system.

Below each cornea lies a lumen, bordered on its sides by two (the primary) pigment cells and at its base by the four cone cells. The primary pigment cells collar the cone cells, one anteriorly and the other posteriorly, and rise to the cornea, so closing off the lumen. Proximal to this arrangement, and extending to the

basement membrane of the retina, lie the bundled eight photoreceptor cells. The photoreceptor grouping is surrounded by six secondary pigment cells each of which is shared by an adjacent ommatidium (Ready et al, 1976). The tertiary pigment cells are found along the proximo-distal axis of the ommatidium in the position of the hexagonal vertices not occupied by the bristles. A tertiary pigment cell is thus found in alternate vertices of the ommatidial hexagonal shape and, as a result, each is shared by three ommatidia (Fig. 1.1). The bristle with its accompanying sensory capability is termed the hair nerve group, the body of which lies nestled between the primary (1°) and secondary (2°) pigment cells below the position where the bristle protrudes from the surface of the eye. The bristle cell group consists of only two cells - the bipolar sense cell and accessory cell (Perry, 1968b), the two other sheath cells (Waddington and Perry, 1960) degenerating during late development. The path followed by the axonal projection from the hair nerve group to the brain has not yet been described, its extremely fine diameter amidst the embracing 2° pigment cells, makes identification very difficult.

Since each 2° pigment cell (2°PC) is shared between two ommatidia then the number attributable to each ommatidium is a half. Each tertiary pigment cell (3°PC), and hair nerve group (HN_g), is shared between three ommatidia and is therefore given the value of 1/3 of a cell. Thus the total complement of cells in each ommatidium is twenty cells:-

$$\begin{aligned}
 &8 \text{ receptor cells} + (6 \times 1/2 \text{ } 2^\circ\text{PC}) + (3 \times 1/3 \text{ } 3^\circ\text{PC}) + (2 \times 3 \times 1/3 \text{ HN}_g) \\
 &\quad + (4 \text{ cone cells}) + (2 \text{ } 1^\circ\text{PC}) \\
 &= 8 + 3 + 1 + 2 + 4 + 2 \\
 &= \underline{20 \text{ cells}}
 \end{aligned}$$

There are two major ommatidial functions - the gathering and channelling of the light, and its reception in the ommatidial body. The cornea and cone cells act as the lens system with the opaque pigment cells optically isolating the ommatidia from each other. However, mutants lacking eye pigment appear to show normal visual abilities (see Pak, 1975).

The light sensitive apparatus lies embedded in the eight photoreceptor cells (R cells) in the core of the ommatidium. Each R cell contains a light sensitive rod or rhabdomere which is a highly folded membranous stack of microvilli (Ready et al, 1976) extending the length of the cell. The membranes carry the light sensitive pigment, retinene, which upon exposure to light is converted, by a reversible reaction, to vitamin A, and so stimulates the nerve impulse. The microvilli are orientated at right angles to the incident light. Each rhabdomere contains parallel orientated microvilli, the microvilli of each rhabdomere being set at an angle to one another. Harris et al, (1976) have shown the eight receptor cells are functionally distinguishable into three distinct groups. Receptor cells R_1 to R_6 inclusive ($R_1 - R_6$) contain the same light sensitive pigment, R_7 another, and R_8 yet another, the three types of pigment being sensitive to different frequencies and thus conferring the R cells with separate visual abilities.

The receptor cells, as with the other cellular components of the ommatidium, exist in a highly organised and regularly spaced pattern. By their positioning in the arrangement, each cell can be identified and assigned a particular number i.e. from R_1 to R_8 . The rhabdomeres

of six of these cells, R_1 to R_6 , form an asymmetrical trapezoid pattern with their cell bodies placed radially (Fig. 1.2). In the distal region of the retina a rhabdomere, borne by R_7 , is placed centrally in this trapezoid arrangement and proximally R_8 provides the central rhabdomere. Hence the rhabdomeric pattern shown in Figure 1.3 can be observed both proximally and distally.

The cell body of R_7 is found between cells R_6 and R_1 and that of R_8 is located much more proximally between R_1 and R_2 (Ready et al, 1976). Both cells bear a cytoplasmic projection into the centre of the rhabdomeric arrangement (Fig. 1.4 and 1.5). The eye as a whole can be divided into dorsal and ventral halves. In each half the rhabdomere pattern is uniformly directed, one half being the mirror image of the other. In each half of the eye the row R_1 R_2 R_3 is positioned anteriorly, pointing in one direction to the poles of the eye and in the opposite direction towards the line defining the pattern inversion. The line of pattern inversion (LPI), which runs in an antero-posterior direction across the centre of the eye, thus separates the eye into two fields, in each of which the ommatidial uniform arrangement is the mirror image of the other (Fig. 1.6).

Proximally, axons project from the receptor cells and penetrate the basement membrane of the retina as ommatidial bundles. The axonic bundles contain two distinct forms of axons. These are the short axons, which terminate in the first optic neuropile - the lamina, and the long axons which pass through the lamina and synapse in the medulla - the second optic neuropile. The short axons originate from the cells R_1 - R_6 and the two long axons are derived from R_7 and R_8 .

Within the ganglia of the lamina and medulla the axons synapse with interneurons. The individual synapses, between axon and interneuron, occurs in distinct cartridges - each axon of the bundle synapsing in a separate cartridge (Meinertzhagen, 1975). The patterning of the nervous projections, in their position within the ganglia, can still be related to the somatic arrangement of their associated receptor cells (Meinertzhagen, 1975).

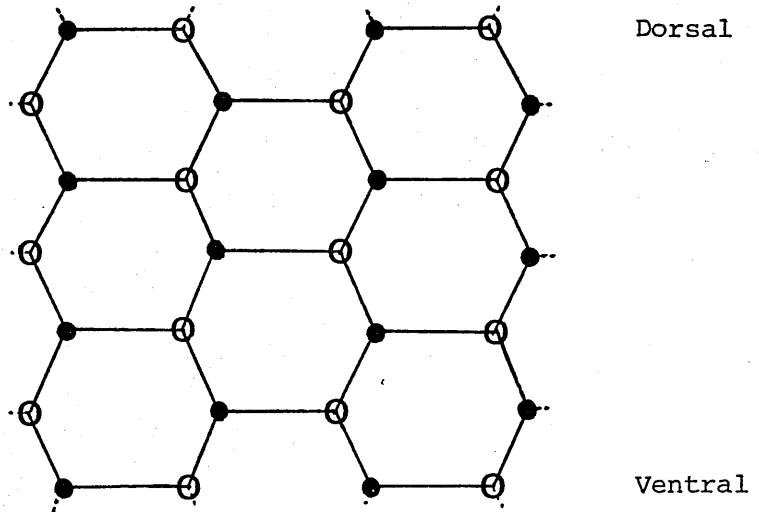


Fig. 1.1 The hexagonal ommatidial array bearing at alternate vertices the hair-nerve group (open circles) and tertiary pigment cells (closed circles).

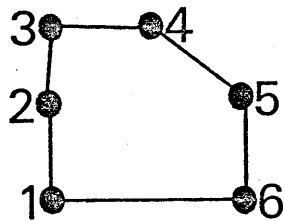


Fig. 1.2. The asymmetrical trapezoid pattern formed by the rhabdomeres of cells R_1 to R_6 . Numbers indicate the cell bearing that rhabdomere. Anterior is to the left.

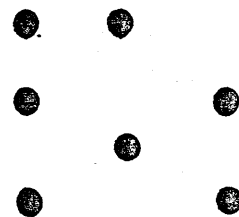


Fig. 1.3. The rhabdomeric pattern found both proximally and distally in the retina.

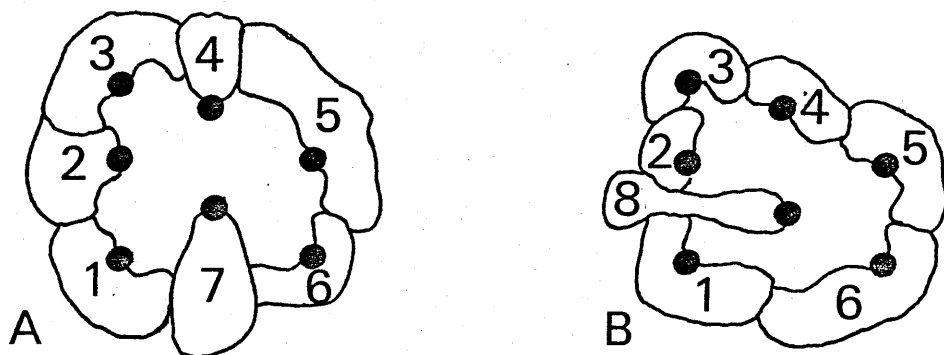


Fig. 1.4. The arrangement of the receptor cells - A distally and B proximally. Anterior is to the left.

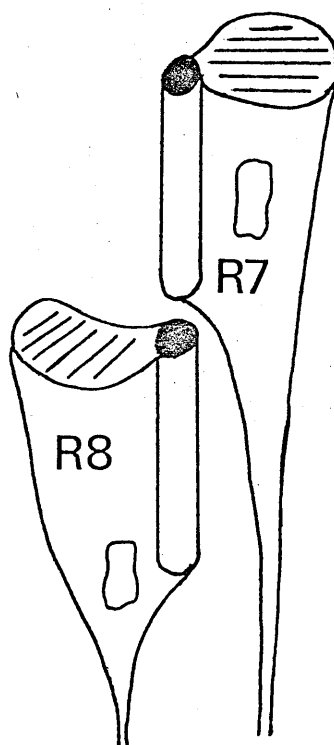


Fig. 1.5. The arrangement of R_7 and R_8 to provide a central rhabdomere both distally and proximally. Distal is to the top.

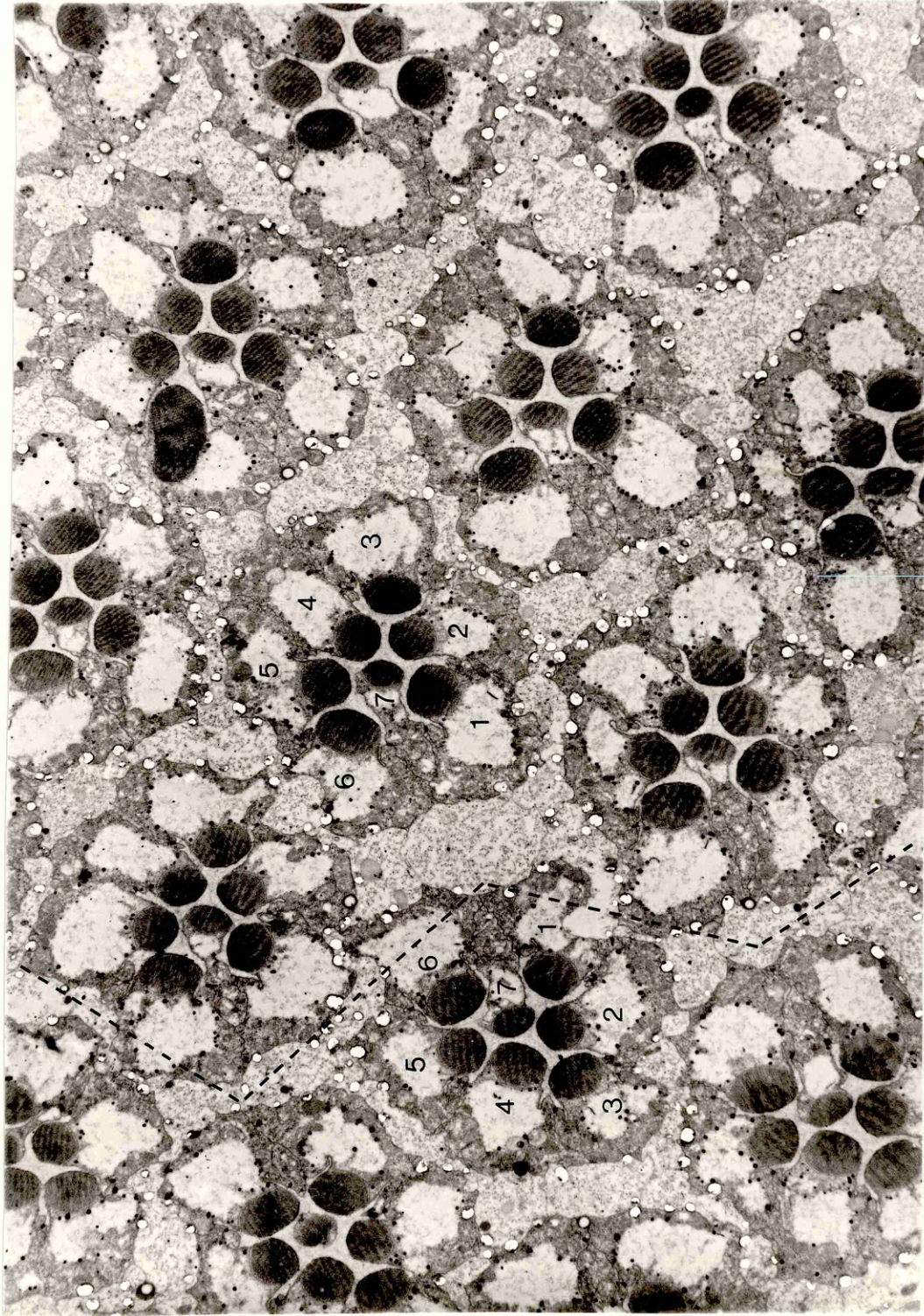


Fig. 1.6 Section through the adult retina. Receptor cells are numbered. Note the mirror image patterning about the LPI (dotted line).

2. POST-EMBRYONIC DEVELOPMENT IN DROSOPHILA

The egg hatches yielding the primary larval instar, two successive moultings lead to the second and third larval instars which are followed by pupation and the eventual emergence of the adult. The egg and first two instars persist for approximately one day each, the third instar lasts two days, and after pupariation four days elapse before eclosion. Thus, the larval development is defined into three distinct stages leading into the pupal phase where larval tissue and structures are converted into the corresponding tissues and structures of the adult by the process of metamorphosis.

In this holometabolous development, the prospective major adult structures are found in the larva as small epidermal specialisations - the so called imaginal discs. In mutants where imaginal discs are absent, development is normal until the pupal period is reached (Shearn et al, 1971) so showing that imaginal discs are not necessary for larval development. The imaginal discs remain undifferentiated until late in larval development and can be considered as isolated from the hormonal system regulating the normal larval epidermal development (Scheiderman and Gilbert, 1964).

Since the subject being dealt with is retinal development the description of the eye imaginal disc in the following section can be used to understand imaginal disc development.

3. EYE DEVELOPMENT

3.1 Larval Period

3.1.1 Introduction

The majority of head cuticular structures of the adult fly are derived from the head, or eye-antennal, imaginal disc. The head disc, as any other imaginal disc, is an embryonic invagination of the ectoderm and is evident in the 16 hour embryo and contact with the larval brain is established during the first larval instar (Campos-Ortega, 1980). The eye stalk, up to the second instar, carries the stemmatal nerve, but as axonal projections develop during the third instar and pupa, the volume increases proportionally with eye development (Campos-Ortega, 1980).

The immature head disc can be considered as a flattened epithelial sac of a single layer of cells connected to the brain by the eye stalk, with the main body of the disc lying on the anterior surface of the brain. The inner surface of the disc consists of cells organised into a columnar epithelium. The outer layer, the so called peripodial membrane is a structure composed of squamous or cuboidal cells (see Sprey and Oldenhave, 1974).

Each head disc gives rise to half the head capsule - the two halves fusing along the mid line of the head (see Milner and Haynie, 1979).

The inner surface of the head disc is known to give rise to the eye and antennal tissues, but the location from where other major head components arise is unclear. Vogt (1946), Gehring (1966), and Ouweneel (1970) published preliminary fate maps and a detailed map was produced

by Haynie (1975) (Fig. 1.7). The experiments performed indicated that the central part of the main body of the head disc gives rise to the retinal field, the antenna being derived from a similar location in the anterior part of the disc, and the head capsule is produced from marginal areas. However, this work was performed on the assumption that the peripodial membrane does not give rise to cuticular structures but degenerates during the early pupal stage (Poodry and Scheiderman, 1970; Ursprung, 1972). Sprey and Oldenhave (1974) have challenged the idea that the thick epithelium is the only constituent of the disc to give rise to adult structures and have cogently argued that a sizeable proportion of the head cuticle is derived from the peripodial membrane and have published tentative fate maps for both the inner and peripodial membranes of the head disc (Fig. 1.8). Milner et al (in proof), by examining head discs undergoing morphogenesis in vitro, have shown that peripodial membrane is definitely involved in cuticle secretion.

Becker (1957) was able to deduce from examination of mosaic patches (see 3.1.5(b)) induced in early development that the compound eye arises from a minimum of two cells. Other such experiments indicate that around twenty cells constitute the prospective eye-antennal field at this incipient period (see Campos-Ortega, 1980). Proliferation and division in the eye disc begins after around 40 hours of development (Madhavan and Scheiderman 1978), and ceases approximately 10 hours after pupariation (Campos-Ortega and Hofbauer 1977). Becker (1957) calculated, from cell counts of squashed second instar head discs, that an 8 to 12 fold increase in cell population occurs in the first instar. Since the head disc cells undergo at least three mitoses during the second instar, then a cell count of 100-150 cells can be regarded as pros-

pective retinal cells at the beginning of this stage (Campos-Ortega, 1980). These cells develop to a population of around 2,000 cells at the beginning of the third instar, which then give rise to the adult ommatidial cells which number (700-800 ommatidia x 20 cells) around 15,000 cells (Campos-Ortega, 1980).

3.1.2 Cellular Patterning in the third larval instar

Approximately 10 hours after the beginning of the third larval instar cytodifferentiation is evident at the extreme posterior of the eye field (Waddington and Perry, 1960). Cells become clustered and the area of clustered cells extends anteriorly as development of the third instar proceeds. Ready et al, (1976) have shown that this patterning process is associated with a deep longitudinal furrow, the morphogenetic groove (MG), which travels across the presumptive eye field in a posterior/anterior direction. Posterior to the groove, clusters of five cells can be distinguished surrounded by unpatterned cells and are termed pre-clusters (Ready et al, 1976). These preclusters lie anterior to a band of dividing cells, to the posterior of which mature clusters of eight cells are evident. The pre-clusters, of five cells, associate with three cells, derived from the mitotic cell band, to produce the mature cluster of eight cells destined to become the receptor cells of the mature ommatidia (Ready et al, 1976). In the area of mature clustering, three different types of cell can be identified, a distal group of four presumptive cone cells, a middle group of eight presumptive R cells, and a proximal group of 1° and 2° PCs (Campos-Ortega, 1980). Posterior to the incipient eight cell clustering, a band of

pycnotic nuclei are evident proximally, which may indicate that cell death is an integral part of the clustering and cytodifferentiation of the cells involved (Campos-Ortega, 1980). However, Fristrom (1969) was able to identify few degenerating cells.

At the mature cluster stage, cell counts have revealed approximately fourteen cells per cluster (Ready et al, 1976). A line of symmetry can be seen traversing the eye field in the region of the mature clusters, travelling in a postero-anterior direction as the anterior tissues develops to the eight cell cluster stage. Clusters in the ventral half of the disc are mirror images of those in the dorsal half, and these opposing patterns meet along a zig-zag border roughly at the equator (Ready et al, 1976). This line of symmetry becomes manifest in the adult eye as the line of pattern inversion (LPI). In the adult eye the LPI is only evident by the pattern inversion itself, but in the third larval instar it seems to be associated with a furrow most evident in the earlier stage. Thus during the third instar, two furrows traverse the eye field; the morphogenetic groove (MG), which lies in the dorso-ventral axis, moves anteriorly across the eye following the furrow associated with pattern inversion. The furrow travels in a direction perpendicular to the morphogenetic furrow (Ready et al, 1976). Because this furrow traces the path which, eventually, will be assumed by the line of pattern inversion, Ready et al, (1976) have suggested that the clusters may be assembled in mirror image form in its wake. However, since it is difficult to identify cells just posterior to the MG, then any polarity of pre-clusters at this stage cannot be established and as a result the opposing images found in the mature cluster field cannot be, directly, traced back to the effect of the furrow.

3.1.3 Morphogenetic abilities of the eye region of the Head Disc

Cells from imaginal discs at varying stages of development can be excised from larvae and surgically implanted into a host, late, third larval instar, which is very shortly about to pupate and begin the process of metamorphosis (Ursprung, 1967). During metamorphosis, the implant will be exposed to the hormones involved in this process and will undergo cytodifferentiation. After eclosion of the host, the disc cells will have differentiated, to a variable extent, into recognisable adult structures (Gateff and Schneiderman, 1975). By increasing the age of the test implants it is possible to determine, for the disc cells, a sequence of developmental abilities (Mindek and Nothiger, (1973); Gateff and Schneiderman, (1975); Campos-Ortega and Gateff, (1976)). This sequence of developmental capabilities can be said to begin with the competence to produce pigment during the second instar and ends with the formation of the ommatidial field. Eyes produced from these implants tend to be smaller than the normal wild type but otherwise perfectly normal. Campos-Ortega and Gateff (1976) were able to show that the youngest (30 hours) implants are able to produce recognisable eye tissue, already contained two distinct cell types - one of them recognisable as R cells and the other 1° or 2° pigment cells. At the end of the second larval instar (45-50 hours) the eye discs were found to be capable of differentiating receptor cells with parallel microvilli. However this ability did not give rise to normal rhabdomers - the microvilli being disordered along the intra-ommatidial side of the plasma-membrane. At this stage also the cone cells can be identified in the adult organ, but no differentiated lenses are present. Implants from the early third instar (approximately 57 hours) are able to differentiate a small number of ommatidia bearing

all the attributes of the normal adult construction, but most ommatidia are only partially formed. Cells in the resulting retinal fields are distinguishable into 1° and 2° pigment cells and often the receptor cells present could be identified as being either of $R_1 - R_6$ or $R_7 + R_8$. The 70 hour implants show a similar situation to the 57 hour situation but with many more ommatidia bearing the full and correctly ordered complement. The 96 hour implants are capable of differentiating a small compound eye which exhibits all the anatomical characteristics of the normal eye. This mature implant also produced in the adult tissue, the line of pattern inversion. A further experiment was performed where tissue from the mature disc was taken from different locations. It was found that only tissue containing the prospective equator produced the pattern inversion in the metamorphosed tissue (Campos-Ortega and Gateff, 1976).

3.1.4 Patterns of mitoses in the eye field of the Head Disc

During S phase a mitotic cell undergoes DNA synthesis for chromosome replication. Thymidine is a specific precursor of DNA, and can be labelled with tritium (^3H) so when supplied to tissue the label will be absorbed by cells replicating their chromosomes. A photographic emulsion can be laid over a section taken from such tissue and will be fogged by radioactive emulsion from the ^3H thymidine, and under microscopic examination a cell underlying the fogged emulsion can be identified as containing the labelled component. This technique is termed autoradiography and is very useful in identification of mitotic activity. Ready et al, (1976) using ^3H thymidine autoradiography discovered two bands

of labelled cells in the third instar eye disc, these being located either side of the morphogenetic groove (MG). The label anterior to the furrow was diffusely distributed whereas the posterior label was confined to a well defined narrow zone extending longitudinally across the disc. Further single labelled cells are seen well behind the posterior band of labelling. These results have been confirmed by the work of Campos-Ortega and Gateff (1976) and Campos-Ortega and Hoffbauer (1977). Examination of adult eyes, which when in the late third instar were pulse-labelled with ^3H thymidine, shows again the two bands traversing the retina. The cells labelled in the anterior zone can be representative of any cell type but in the posterior band the cells $R_{2,3,4,5}$ and 8 are never labelled. Thus it can be concluded that the cells $R_{2,3,4,5}$ and 8 reach their final mitosis in the anterior wave of division. Ready et al (1976) examined disc and pupal eye tissue after pulse labelling and here again only $R_{1,6}$ and 7 , of the photoreceptor cells, showed the label in the posterior band. Campos-Ortega (1980), examining heavily labelled eyes, was able to conclude that not only $R_{1,6}$ and 7 are produced from the posterior wave but also cone cells and 1° and 2° pigment cells. Of course, when the anterior band of label is examined, cone cells, 1° and 2°P cells, and $R_{1,6}$ and 7 are sometimes found labelled along with $R_{2,3,4,5}$ and 8 . This is due to the fact that their precursor cells underwent division in the anterior wave and thus took up the label. Development then continued without the label being supplied. When the cells labelled then went into the second (posterior), $R_{1,6}$ and 7 generating division, some of their progeny carried the label absorbed in the earlier division.

Taking these autoradiographical studies as a whole it can be said that during the third larval instar, ommatidial assembly takes place in the following way. Firstly a mitotic division takes place generating a pool of cells present in which are the $R_{2,3,4,5}$ and 8 cells which will not divide again. The cells then experience the constriction of the MG, after which the pre-clustering pattern of the five cell ($R_{2,3,4,5}$ and 8) grouping is formed. A second mitotic wave now occurs generating the remaining cells required for ommatidial assembly. Campos-Ortega (1980) has identified a small amount of division taking place after the time of the two major division phases, and suggests that this is due to a few remaining secondary pigment cells arising from isolated mitoses. The reason, or function, of the MG in this developmental process is as yet obscure. It cannot be established whether the $R_{2,3,4,5}$ and 8 become associated prior to the MG or whether the steric and mechanical constraints imposed by the furrow are responsible for bringing them into intimacy, thus forming the five cell pre-clustering patterns.

If the third larval instar eye disc is viewed as a whole, then the antero-posterior axis can be considered as one of time. Travelling from the anterior in a posterior direction, one can observe - division, MG, pre-clustering, division, and finally mature clustering. The distance between each of these happenings is a physical separation which indicates the temporal sequence in each patch of tissue. These processes begin in the posterior of the disc, and progress anteriorly, giving a posterior to anterior sequence of development.

Experiments have been performed which confirm that a developmental process traverses the eye field in a posterior to anterior direction. van Breugel et al, (1975) injected actinomycin D (which prevents DNA transcription) and fluorouracil (which prevents RNA translation) into third instar larvae. Both of these treatments produced a similar effect in which a band of disruption, in the dorso-ventral plane, occurred. The relative antero-posterior positioning of the band was directly related to the age of the instar injected.

Poodry et al, (1973) and van Breugel et al, (1975), using the mutations shibire and Notch respectively, by exposing third larval instars to brief increased temperature shifts were able to elicit a similar band of retinal disruption as found in the experiments described above. It is also known that these results can be produced by X-irradiation of larvae during the same period.

We know that two waves of division are present in the eye region during the third larval instar and if the temperature shift experiments were affecting this process then two bands of disruption would be expected to be manifest in the adult eye. Being aware that the treatments interfering with RNA transcription and translation, essential mechanisms in cell differentiation, produce the same result as the temperature shift experiments on the aforementioned mutations, then it is not unreasonable to assume that these experiments are all affecting cells undergoing differentiation. This is quite an interesting indication in that it tends to conflict with the process of ommatidial assembly as previously described - Ready et al (1976) have shown five of the receptor cells to be clustered before the remaining three cells

required for the mature receptor cell bundle have gone through their terminal division. The physical pre-clustering indicates that these five cells have undergone a certain amount of differentiation, and knowing that at this stage the remaining three cells are yet to divide and hence cannot be differentiated, then one might expect two waves of disruption to be detectable.

All these results indicate that the development of the eye field is a highly organised and precise temporal sequence of events, at certain stages of which there is vulnerability to disruption.

The patterns of mitosis occurring in the retinal field have been described as they have been interpreted by the various authors mentioned. The anterior mitotic wave has been described as traversing a pre-existing retinal field. However Nowel and Shelton (1980) have challenged this view and argue that the apparent behaviour of the anterior mitotic wave is also consistent with an anteriorly placed zone of proliferation generating retinal tissue to its posterior. The nature of the anterior mitotic wave will be discussed in more detail later.

3.1.5 Cell lineage and restrictive boundaries

Clonal analysis is the study of cellular systems in which a population of cells can be identified as sharing a common progenitor cell. The technique involves generating homozygous tissue for one or a number of loci in a heterozygous background. The situation where two genetically distinct tissues are found in the same animal is termed

mosaicism. There are two distinct methods of producing mosaics; chromosome elimination, and mitotic recombination. Chromosome elimination occurs when an X chromosome is lost from a cleavage nucleus in female tissue resulting in male and female cell populations - a so termed gynandromorph. Mitotic recombination, which occurs spontaneously at low frequency can be induced at much higher frequencies by varied techniques - the most commonly used inducer being X irradiation. For detailed explanation of the production of mosaics the reader is referred to Postlethwait (1978).

(a) Compartments and homeotic genes

The concept of the compartment was first formulated by Garcia-Bellido et al, (1973) whilst studying the developing wing imaginal disc of Drosophila. It has been found in clonal analysis of Drosophila structures that clones induced early in development can spread over several segments, but clones initiated later in development were restricted to a single segment (Bryant and Schneiderman 1969). It was also noticed that clones marked early in development might include both thorax and wing, or both dorsal and ventral surfaces of the wing, but clones induced later were restricted to one or other of these regions (Bryant, 1970). From these results it became clear that in these structures there was some kind of clonal restriction operating particularly at relatively later times. The clonal analysis technique was used with a variety of mutations where the phenotype could easily be distinguished from the wild type background. However, irradiation at early stages, due to the fact that there are fewer target cells but with many divisions ahead of them, produce a few,

large, clones. Later inductions result in a higher frequency of smaller patches because of the greater number of target cells but their prospective divisions being limited. Under these circumstances the recognition of the boundaries restricting clonal expansion proved difficult especially where no morphological boundary was present to serve as a guideline. This difficulty was overcome by using a dominant Minute (M) mutation, which in the heterozygous condition causes slow growth and a low mitotic rate in the cell. Clones now induced (Fig. 1.10) bore marker genes such as white (w) but also lacked the M genes and were, as a result, able to develop at a much faster rate than the background tissue. Morata and Ripoll (1975) discovered that wild type clones could grow up to twenty times larger in a Minute background than in the normal situation. Garcia-Bellido et al, (1973) investigated the growth of such clones in the wing discs and found that even these extremely rapidly growing cells respected certain demarcation lines provided that they are initiated after a certain time. Clones induced in the blastoderm (Wieschaus and Gehring, 1976) can extend from second leg to wing, or third leg to haltere, but clones in flies, irradiated during 24-96 hour period, stopped at the borders of the wing disc (Garcia-Bellido, et al, 1976) or leg disc (Steiner, 1976).

The area defined by the boundaries restricting clonal expansion have been termed compartments. Perhaps the most striking example of these restrictions is the anterior-posterior boundary which lies in the wing. This line of restriction separating the anterior from the posterior dorsal wing compartments lies close to, but not on, vein IV. The M⁺ cell clone produces a patch which may fill 30 per cent of either anterior or posterior compartment, and may have hundreds of cells on the compartment border but never crosses it (Garcia-Bellido et al, 1973).

(b) Clonal analysis and indications of cell lineage in the eye

(i) Early cell lineage

Becker (1957, 1966) was the first observer to present evidence indicating both developmental restrictions within the developing head disc and a cell lineage relationship in the formation of the compound eye. Examining clones induced in embryo and early larval stages, he found that the clones had a border at the middle of the eye which resembled very closely the LPI (see Chapter 1.1). He was able to conclude that, for the most part, cell lineages of the upper and lower halves of the eye seem to be separated. In addition to this major central border line he was able to identify less marked lines of restriction particularly in the ventral half of the eye. These lines delimited certain sections in the eye and it was found that a clone, of the size of a single section, would be produced in the adult eye if irradiation took place around the moult between the first and second larval instars. He was able to estimate that each cell of the twenty, or so, present in the prospective eye field of the late first instar, would give rise to an average of forty ommatidia. The sections were not clearly defined in the adult eye, they were more a series of areas revealed by the bunching of borders evident when tracings from many eyes were superimposed upon one and another. Thus the clone boundaries indicated, diffusely, the presence of weakly restrictive areas of constraint in development. This suggested that at the end of the first larval instar a prospective cell in the imaginal disc seems to have been assigned the formation of a certain section of the eye.

To study the lineage restrictions of the mid-eye, and sectoral, boundaries described by Becker (1966), Campos-Ortega and Waitz (1978)

carried out clonal analysis of retinal development. By using the Minute mutation, they attempted to see whether a clone of \underline{M}^+ cells in a \underline{M} background would respect the postulated boundaries. The large \underline{M}^+ clones tended to have very irregular borders and ran across the eye without following any particular direction. The majority of clones, however, bore at least one straight edge, and five straight lines, three horizontal and two predominantly vertical, have been recognised as being boundaries preferentially respected by \underline{M}^+ clones. The positions of three out of five of these restrictive lines corresponded quite accurately with those of the dorsal and anterior boundaries described by Becker (1966). Another interesting result from this work (Campos-Ortega and Waitz, 1978) was the detailed histological study of the relationship between the mid eye clonal boundary and the LPI. Electron micrographs revealed that they do not invariably coincide, but they may deviate from each other by one or two ommatidial rows (a finding confirmed by Ready et al, 1976). Hence the dorsal and ventral halves of the eye are not compartments and the LPI is a weak restrictive boundary, but the cells of the ventral and dorsal halves of the eye may have separate clonal origins.

Campos-Ortega and Waitz (1978) were able to generate by irradiation eyes with abnormally positioned LPI's from some female larvae of the cross:-

$$M(2) \ 0^{SP}/Bin \ sn \ X \ y \ w \ f \ 36/Y; \ ey^2/ey^2$$

The phenotypes consist of abnormalities in the ventral third of the eye. The phenotype varies from a disruption of 4-6 ommatidial rows below the LPI, to the extent of completely affecting the ventral third of the eye. Examination of third instar eye discs showed cell death within the ventral half of the eye field before the

groove has passed i.e. prior to pattern initiation.

Another major effect of the phenotype is the positioning of the LPI - it starts posteriorly in a similar position to the normal eye, but soon drops down some rows and then runs irregularly to the anterior of the eye. Clones induced in these eyes show similar boundaries with respect to the middle line found in normal eyes. This result, in association with earlier indications, cogently indicates that the LPI does not act as a restrictive boundary for clones growing in the ventral and dorsal halves of the disc. It may be, however, that the mechanism that establishes the LPI and the mid line restriction is one and the same.

Ready et al, (1976) proposed that the equatorial groove, traversing the third instar eye field ahead of the MG, may well be responsible for the establishment of the LPI. This groove may well act as a physical constraint preventing ventral cells from mixing with dorsal cells. It is known, however, that cells that cross the LPI, do so generally during the third instar (Campos-Ortega and Waitz, 1978) and since the groove is present very early on in the instar, then the cells cross the LPI whilst this constriction is present. Campos-Ortega and Waitz (1978) have shown that the line of restriction's effectiveness increases with the age of the third instar but the equatorial groove shows constant characteristics during the instar, diminishing only in extent as pattern formation is established.

To prove conclusively the presence of a delimiting developmental restriction line, and hence the evidence for a developmental compartment, requires results obtained from mutations demonstrating the genetic control of the compartment. In the eye such genetic evidence is lacking

and as a result the stronger lines of developmental restriction found by Becker (1966 and 1957) and Campos-Ortega and Waitz (1978) cannot, as yet, be said to be defining compartments.

The descriptions of cell lineage given above, have been with reference to the prospective eye region of the head disc, whereas the disc as a whole contains presumptive eye tissues and antennal tissue (the other head structures can be ignored for our purposes at this stage). Other imaginal discs may be considered singular in their major developmental product, e.g. they give rise to a wing or a leg, but the head disc produces both the eye and antenna. Hence, is the head disc a single disc, or is it the result of an evolutionary fusion of earlier separate discs? To discuss this in detail necessitates evidence derived from the analysis of dipteran, and more generally insect, evolution. This subject involves debate such as how many ancestral segments are fused to form the head complex. A succinct description of this problem is presented by Morata and Lawrence (1979). In the same paper, using homeotic mutations (see 3.1.5(a)) such as aristapaedia, they were able to demonstrate that a restrictive boundary exists within the developing antennal tissue. Although the segregation resulting was established during the larval period, (other compartments such as in the wing being established during the blastoderm), they concluded that it should be afforded full compartmental border status. The head disc is, thus, separated into two compartments. This then has shown the existence of compartments in the head disc which had not previously been demonstrated. If the disc is to be considered as ancestrally single, then the head disc has, although established somewhat later in development, normal compartmental status. But if the head disc is to be viewed as the fusion of two discs,

then the eye disc as yet lacks a genetically proven compartmental separation into posterior and anterior.

(ii) Late cell lineage

One of the great debates of contemporary embryology is whether cellular differentiation is determined by the cell's lineage i.e. the cell behaving in response to genetic commitments made by its ancestors, or whether the cell, at the time of differentiation, receives positional information from surrounding tissue to direct that differentiation. It is generally regarded that both processes occur, but which of the two plays the major role has yet to be determined. Data available indicates that differing phyla tend to place differing emphasis on the two.

The highly precise and repetitive structure of the compound eye of dipterans provides an excellent subject for examining a developmental process that occurs in insects.

The very precision of the compound eye led Bernard (1937), and Kuhn (1965), to postulate that it developed by precise cell lineages. Studies of mosaicism in the compound eyes have led to the negation of this proposal (for Drosophila see Hanson et al, 1972). If an ommatidium develops directly from ommatidial 'mother' cells (a cell from which all the cells of an ommatidium develop), as the cell lineage theory suggests, then an ommatidial mother cell marked by mitotic recombination will give rise to an ommatidium containing cells all carrying this marker. Ready et al, (1976), using the sex linked cell autonomous recessive mutation white (w), induced clones at the time of the late

first instar when the eye primordium comprises approximately twenty cells. They examined the adult eye and found ommatidia of mosaic constitution i.e. the cells of these ommatidia were of two types - white and wild type. This result, thus, overturns the theory that precise cell lineage, mother cells producing the ommatidia, is acting. However, cell lineage may still be important i.e. the receptor cells may develop from a receptor cell mother cell, and the pigment cells from another similar lineage. To clarify this situation, Ready et al (1976) examined many mosaic ommatidia and were able to conclude that, in an ommatidium, any pair of cells could show different phenotypes e.g. - the two primary pigment cells can be different, similarly a secondary pigment cell need not be of the same clone as any neighbouring pigment or receptor cell, and any R cell can display a different phenotype from any other. Thus, the idea of cell lineage being an important part of the development of the eye of Drosophila is largely negated. This result led Ready et al, (1976) to describe the ommatidial assembly as being analogous to chemical crystal formation, the cells, being recruited from a pool of those available, into a growing lattice system.

Hofbauer and Campos-Ortega (1976) performed similar experiments to those of Ready et al, (1976), and yielded confirming results. Suspecting that cell lineage may still play a part in retinal assembly, they then performed a further scrutiny of their data. They argued that it is possible that some of the R cells are linked by ancestry, not just by the fact that sister cells from a late division will not migrate from each other appreciably, and as a result may tend to be recruited into the same ommatidial cluster. By statistical analysis, they were able

to show by examining mosaic ommatidia, that the receptor cells could be divided into two groups - R_{2-5} and $R_{1,6+7}$ (R_8 was ignored due to difficulty of scoring). It was found that pairs of receptor cells showing the white phenotype were present in a greater proportion when they were members of one of the two groups mentioned, and in a significantly less number when the two contained a member of each. This indicated clonal relationship can be explained by, and also confirms, the patterns of mitosis found in the third larval instar by Ready et al, (1976) (3.1.4). The $R_{2,3,4,5+8}$ are known to reach their terminal division one mitosis earlier than $R_{1,6+7}$. These two groups of cells are thus mitotically isolated and not clonally distinct as the results of Hofbauer and Campos-Ortega (1976) indicated. Since it is known that any R cell in an ommatidium may or may not bear the same lineage as any other, then the tendency to produce two groups in clonal analysis is a result of their staggered terminal mitoses.

The clonal experiments hitherto described deal with large clones i.e. the first larval instar, with few prospective retinal cells, being irradiated. Campos-Ortega and Hofbauer (1977) have argued that cell lineage, being involved in the cellular determination, is not ruled out by this methodology. They presented results derived from larvae irradiated during the third larval instar. As explained earlier, the later the irradiation then the greater the number, but the smaller the size of the clones produced (3.1.5(a)). From their results they were able to deduce that a segregation of the cellular population does take place between the second and third instars. Additional information from this analysis indicated that a precursor of an R cell is able to give rise to eight or possibly nine such cells.

Lawrence and Green (1979), have criticised and produced contrasting results to the aforementioned work of Hofbauer and Campos-Ortega (1977). They maintain that the experimental technique was fraught with problems. The clones induced by Hofbauer and Campos-Ortega (1977) were marked by the sex linked mutations white and rdgB against a heterozygous, phenotypically wild type, background. Receptor cells do not contain large amounts of pigment and to establish its absence often proved difficult. Because irradiation was during the third larval instar, then many clones were induced, and as a result difficulty was experienced in distinguishing one clone from another. The operating criteria adopted by Hofbauer and Campos-Ortega (1977) was to regard a patch of mutant tissue as representative of a single clone when it was restricted to seven ommatidia i.e., one central ommatidium and its immediate neighbours. They also explained the presence of ommatidia containing both receptor and pigment cells in mutant patches as not negating the late separate lineage process. They preferred to interpret the presence of such ommatidia as resulting from the mixing of two distinct clones. As has been explained, much of the confusion arising from this work pertains to the difficulty in distinguishing a clone from its neighbour. Lawrence and Green (1979) adopted a technique which ameliorated this problem. Instead of producing a mutant white clone against a wild type background, the method of Stern (1968) of inducing white⁺ cells in a phenotypically white background was adopted. The technique involved generating, by X-irradiation, recombination within the white locus. Two different white alleles are arranged in trans, such that mitotic recombination can result in the formation of a wild type chromosome. Because the required recombination is limited to the location of a single locus, then the frequency of that recombination,

and of the clones accruing is correspondingly low (Fig. 1.11). Lawrence and Green (1979) established the frequency of one clone per thirty eyes, the problem of confirming the singularity of a clone was thus resolved. This research also examined the phenotype of the R_g cell, an investigation Hofbauer and Campos-Ortega (1977) failed to carry out. Examination of clones induced during the very late mitoses of retinal development, showed label in both pigment and receptor cells in diverse combinations, and thus provided cogen evidence for the lack of rigid cell lineages acting in development. Because of the low frequency of clone induction many eyes needed to be scored, creating a formidable amount of work. To produce conclusive results many more clones need to be scored. But the results yielded ruled out simple cell lineage such as the separation into receptor and pigment cell lines, and also more obscure situations such as where a particular, or group of, pigment cells bear common ancestry with a particular, or group of, receptor cells. To disprove conclusively the lack of separation of cells into particular lineages during development in the retina requires considerably more investigation of such low frequency clones.

One of the major problems facing the research of elementary particles, is that the physicist suspects that the behaviour of particles, such as the electron, relates only to the prevailing experimental conditions and not its normality. In the biological field awareness of experimental interference is also essential. The methods so far related involve the use of radioactive DNA bases and also exposure to direct X-irradiation. The effect of X-rays, in particular, is known to produce cell disruption and commonly death (Haynie and Bryant, 1977) and the interpretation of results from techniques utilising such agents, must be viewed with circumspection.

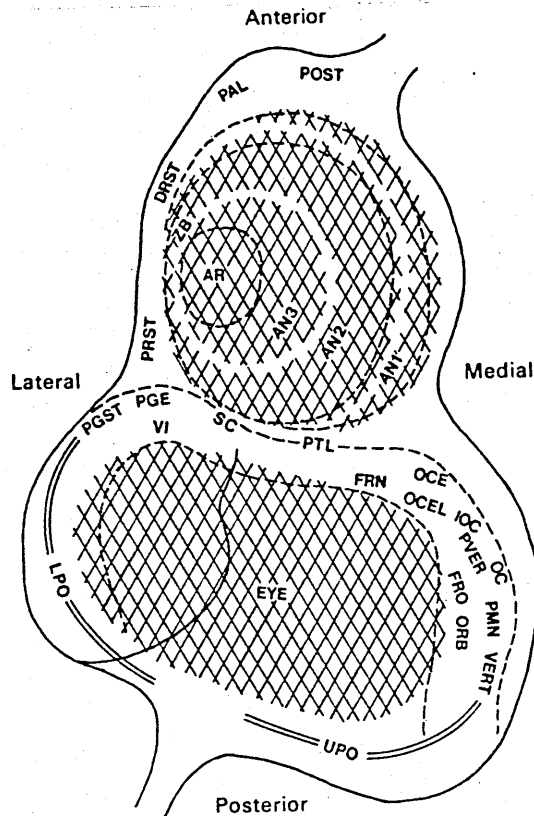


Fig. 1.7. Fate map of the eye-antenna disc. (From Haynie, 1975.) POST: postocipital sensilla; PAL: palpus; DRST: distal rostral sensilla; PRST: proximal rostral sensilla; PGST: postgenal sensilla; LPO: lower postorbital bristles; PGE: postgenal bristles; VI: vibrissae; SC: shingle cuticle of lateral ptilinum; PTL: ptilinum; ZB: zahnborsten (thorn-like bristles of second antennal segment); AN1, AN2, AN3: first, second, and third antennal segments; AR: arista; FRN: frontal bristles; OCE: ocellus; OCEL: ocellar bristle; IOC: interocellar bristles; PVER: postvertical bristle; OC: occipital bristles; PMN: premandibular bristles; FRO: fronto-orbital bristles; ORB: orbital bristles; VERT: vertical bristles; UPO: upper postorbital bristles.

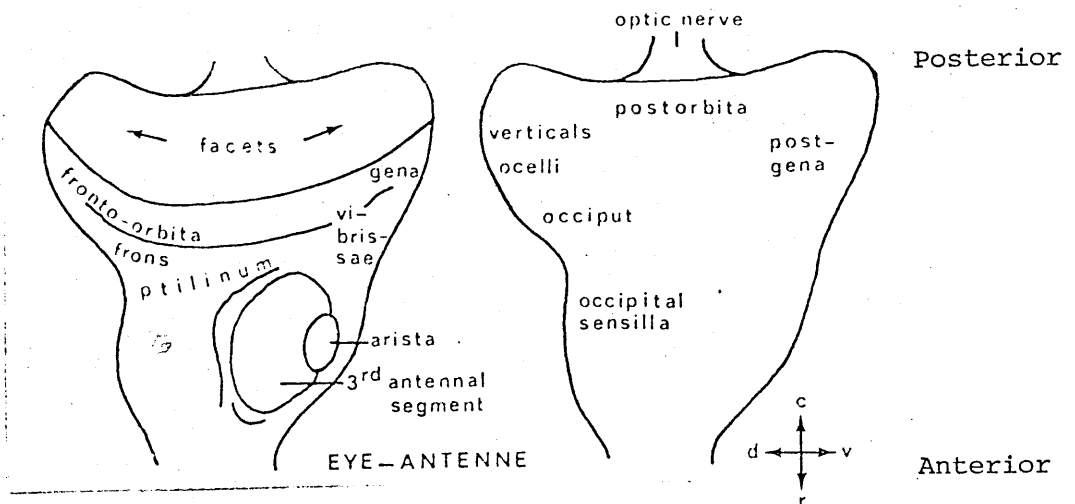


Fig. 1.8. Tentative fate map of the inner (left) and peripodial (right) layers of the head disc. (After Sprey and Oldenhave, 1974.)

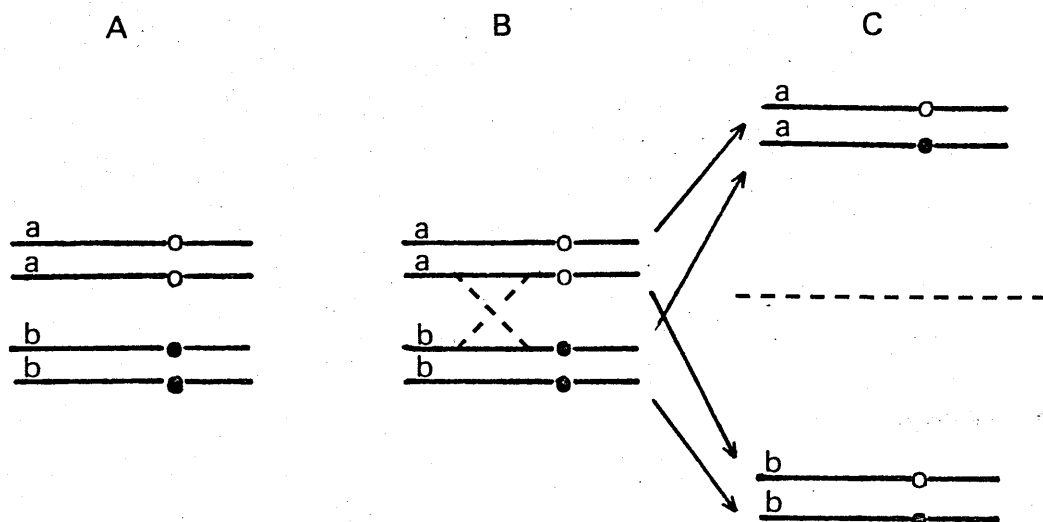


Fig. 1.9 Mitotic recombination. A: cells heterozygous for a particular locus are subjected to X-irradiation during prophase; B: a recombination event between non-sister chromatids is induced; C: completion of the mitotic process results in daughter cells of homozygous condition.

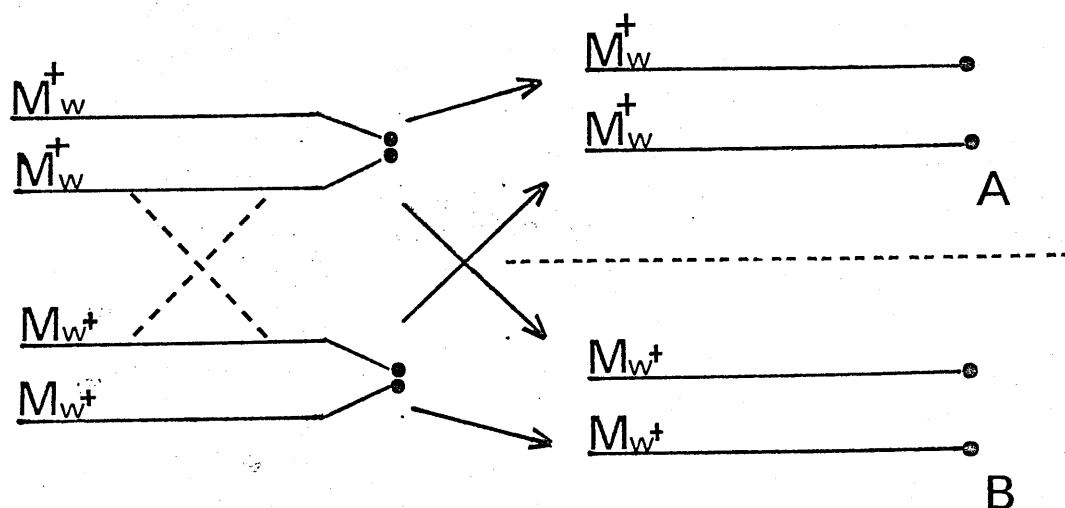


Fig. 1.10. Mitotic recombination within a cell heterozygous for the Minute and white loci resulting in daughter cells of phenotype A: white; B: Minute.

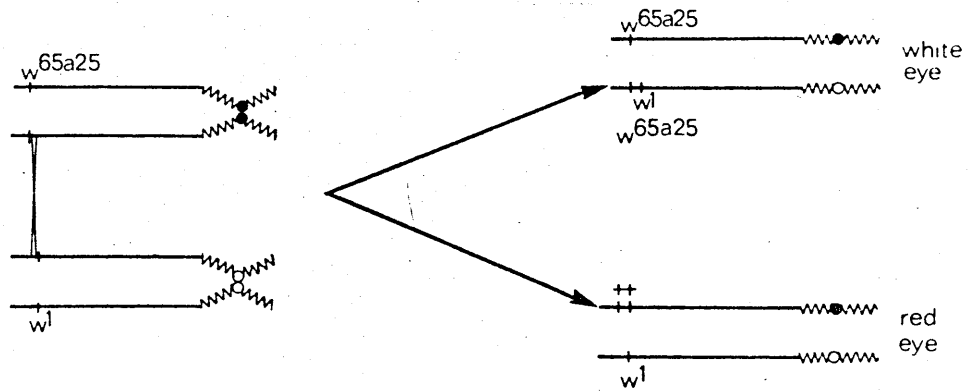


Fig. 1.11. Mitotic recombination within a cell heterozygous for two white alleles. Recombination within the white locus can generate a white⁺ chromosome. (After Lawrence and Green, 1979)

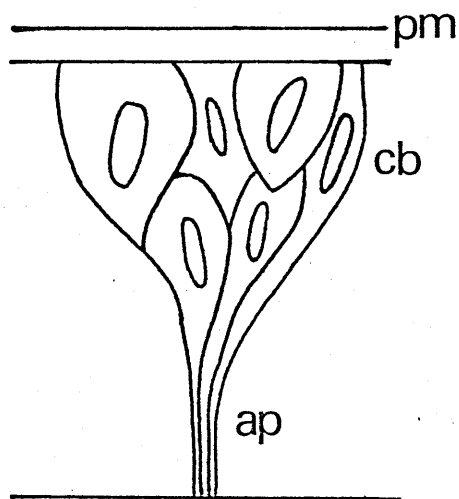


Fig. 1.12. The 'spring onion' appearance of the receptor cell bundle in the late, third, larval instar. (After Waddington and Perry, 1960). pm: peripodial membrane; cb: cell bodies; ap: axonal projections.

3.2 Pupal Eye Development

3.2.1 A review of the mature third instar eye region

Waddington and Perry (1960) described the mature, eight cell clusters as appearing like bundles of spring onions, the swollen cell bodies corresponding to the onion bulbs (Fig. 1.12). Ready et al, (1976), by serial sectioning, were able to describe the cellular construction of the eye field of the late, third larval instar in more detail. Each cluster contains a central cell, contacting which along the majority of its length lie the remaining seven. Posteriorly from each mature cluster runs a bundle of eight axons into the optic stalk. Seven of the axons are arranged around a central projection in positions relatable to the cells within the cluster of the cell bodies. The cone cell nuclei can be found distal to the cluster and the pigment cell bodies surround the axon bundles proximally.

3.2.2 Receptor cell development during the pupal stage

The majority of the following is taken from Perry (1968a). A day after pupariation, receptor cells become slightly broader and localised densities are evident on adjacent cell membranes which show a small amount of folding and interdigitation. More proximally, less differentiation is evident, microtubules being mainly found in the axonal projections and distal tips of the cells. By 40 hours, after pupation, rhabdomere formation can be said to have begun - shallow folds can be seen, interdigitating along the central cell margins, with opposing cells of the group (Perry, 1968a). The eighth receptor cell is located in a more proximal position than the remaining seven, rhabdomeric formation also taking place in this cell. Microtubules

are intimately associated with rhabdomere formation. They can be seen in high concentration immediately adjacent to the folded membranes (Perry, 1968a). In the adult situation, the rhabdomeres are projected from the main body of the cell (more pronounced in $R_7 + 8$) into the central lumen between the R cells. The withdrawal from this central axis begins a few hours after the 48 hour stage and the microvillar projections are thus pulled away from each other, and as a result can be seen more clearly (Perry, 1968a). Waddington (1962) has described special cell junctions which unite adjacent cells in the region of the ommatidial lumen. They consist of high concentrations of granular material closely bound to the respective cell membranes, with a less dense material present in the inter-cellular gap of approximately 15nm. Perry (1968a) has interpreted these junctions as being, or very similar to, the intermediate junctions (zonula adhaerens), described by Farquhar and Palade (1963), in vertebrate tissue. Perry (1968a) has noted the presence of small flattened vesicles in the cytoplasm around the developing rhabdomeres. Although vesicles can be seen fused to the microvilli, later in development, there is little evidence to suggest that these vesicles are involved in fusion with the microvilli at this stage. Between the 48 and 72 hour period, the high concentration of microtubules, present in association with the incipient rhabdomeres, tends to become disorganised and lower in density (Perry, 1968a). It is during this period that the major increase in eye region depth occurs, an approximately four fold elongation from 25-110 μ in the proximal/distal plane. Accompanying this depth increase is the rapid growth of the R cells. After this elongation process, the microtubules are distributed throughout the cytoplasm and show no association with the rhabdomeres (Perry, 1968a). The cell junctions, mentioned previously,

do not display any further differentiation, but the cytoplasm adjacent to the rhabdomeres becomes noticeably short of organelles and structural materials - its composition being sparsely granular (Perry, 1968). During this growth, the lateral extent of the microvillous projections remains unchanged (approximately $5\mu\text{m}$) but during the fourth day of pupal development their length increases and forms the hexagonal packing arrangement evident in cross sections of the adult eye. The microtubules, once highly associated with rhabdomeric construction, now become absent from the main body of the cell, their only persistence being as neurotubules in the axonal projections.

3.2.3 Cone cell development during the pupal stage

At the onset of pupation, the cone cell bodies are found distal to the receptor cell group. They remain relatively undifferentiated during the first half of pupal development. The cone cells are responsible for the secretion of the extra-cellular, corneal lens and from 40-77 hours after pupariation their function is directly involved with this. After the 40 hour stage the cone cells bear distally, microvilli. At the tips of these, small dense patches are identifiable on the external surface (Perry, 1968a). After a further eight hours development, these patches have developed into a continuous, cuticulin covering of the cone cell distal surface. Dense granular material then accumulates against this covering, following which fibrous tissues is laid down to eventually produce the laminated hemispherical lens (Perry, 1968a). Microtubules, known to be important in rhabdomere formation, are also highly involved in cone cell differentiation. During the external secretion of the lens, microtubules, in a web like arrangement, can be seen lying parallel to

the distal axis of each of the four cells. Their location is limited to an ommatidially central position where the four cells are in contact. On the last day of pupation, the proximal boundaries of each cell are pulled away from the lens, thus shortening the cells and causing a lumen between the cell and the lens - the so termed pseudocone. The boundaries are withdrawn more from the central than lateral position. After this shortening, the microtubules remain in a similar position and after eclosion a further retraction takes place causing a squashing of the nuclei and a more intimate covering of the underlying rhabdomeres.

Approximately two weeks after eclosion, the adult eye still displays microtubules placed centrally in the cone cell group, but after a month they have been broken down, their position being replaced by small irregularly shaped vesicles (Perry, 1968a).

The cone cells show a number of surface specialisations during the pupal, and later, development. Their lateral boundaries, after retraction from the secreted lens, are thrown into many folds and become highly interwoven. Septate desmosomes develop between cone cells and underlying receptor cells are of the adhering zone type found between adjacent receptor cells.

3.2.4 Pigment cell development during the pupal stages

Little appears to be known about the development of the pigment cells during the pupal phase - our knowledge is largely limited to descriptions of pigment granule formation. Shoup (1966) has stated that the essential adult arrangement of cells is established by 72 hours after pupariation,

but the processes resulting in this situation are not described.

Perry (1968a), examining pigment granule formation, was able to observe cytoplasmic granules of varied morphology in the basal region of the retina - they were evident between 24 and 40 hours after pupation, but by 48 hours, when true pigment granule formation had been initiated (Shoup, 1966), they had disappeared.

Shoup (1966) was able to identify three types of pigment granules, and a fourth characteristic of colourless mutations, in later (after 48 hours from pupation) development. The Type I are small dense granules found in secondary pigment cells around 48 hours after pupation and tend to be closely associated with Golgi vesicles. The pigment is assumed to be assembled in the Golgi, released into the cytoplasm as membrane-bound vesicles, and then differentiate into mature pigment granules. The primary pigment cells reveal the presence of the Type I granules some 12 hours after they appear in the secondary pigment cells and as a result by 60 hours after puparium formation the secondary pigment cells contain much larger granules than the primary. By 78 hours after pupation the Type I granules had reached their final size and had become electron opaque. Shoup (1966) was able to conclude that the Type I granules observed with the electron microscope correspond to the brownish ommochrome pigment evident in light microscope sections.

The Type II granules are found only in the secondary pigment cells and are believed to contain the pigment drosopterin (Shoup, 1966). They first appear at about 60 hours after pupation and show a filling and darkening process during maturation but little increase in size.

The Type III pigment granule has occasionally been identified between 60 and 70 hours after puparium formation but is more strongly associated with mutations such as brown and vermilion. These granules are found in the secondary pigment cells and show a close proximity to Golgi vesicles and as a result are thought to be abnormal Type I granules (Shoup, 1966).

The granules described in the basal region of the retina during early pupal development may well be the Type IV granules (Perry, 1968a) but Shoup (1966) has only identified them in eye colour mutations such as white (w). They appear in w eyes about 54 hours after pupation and have lysosome characteristics and because they are found only in the secondary pigment cells they are thought to be related to abnormal pigment development. It is believed that the Type IV granule represents accumulations of aberrant pigment precursors which are prevented from undergoing normal development (Shoup, 1966).

4. GENETIC MUTATIONS AND THE ANALYSIS OF EYE DEVELOPMENT

4.1 Introduction

As has been explained, the developmental process resulting in the production of the adult eye have not yet been fully described. Genetic mutations can be used to examine the genetic control of certain developmental pathways, but their major value, at the moment, is in the examination of the aberrant development they induce, and the information derived by comparison with the wild type situation.

Mutations occur spontaneously but are commonly induced by the process of mutagenesis. The usual method adopted is to expose immature male gonads to a mutagen delivered to the animal in food such as sucrose solution. The mutagen, when in proximity to the gonads, causes base pair rearrangement in the chromosomal DNA, and as a result phenotypic mutants can be produced (see Ransom, 1983).

Many mutations are recessive i.e., the wild type phenotype will be expressed when the genotype is heterozygous for the locus in question. The mutant recessive phenotype is only expressed when both loci carry the mutant gene. Dominant and semidominant mutants are relatively rarer with respect to the wild type gene. Sterility can often result from the homozygous recessive condition, and in such situations the mutant gene is carried in a specially 'balanced' heterozygote; only the mutant homozygote and heterozygote being viable genotypes. Lethality

is another problem encountered with the mutant homozygote, here the mutant can only be examined as viable tissue induced in the wild-type background (see 3.1.5).

There are a considerable number of mutations affecting the development of the eye, a limited number of which are extremely specialised in their effect - disrupting, or eliminating, a particular cell type, or developmental process. Investigation of such mutants can reveal the time of action of the locus and its effect upon tissue differentiation (see Campos-Ortega, 1980).

One of the major problems, encountered during experimentation with such mutants, is to adequately distinguish the primary from secondary effects i.e. to recognise the effect of cell autonomy from non-cell autonomy:-

[A locus can be said to be cell autonomous if a gene occupying that locus in a host cell is able to determine the phenotype controlled by that locus in the cell regardless of the phenotypes of the surrounding cells and tissues],

e.g. a locus may be cell autonomous and relates to a particular, or cell type, and the cell phenotype will be mutant when the genotype is homozygous. However other cells, to which the locus does not relate, which develop in association with the previous cell type, may show aberration in development due to the fact that the aforementioned cell type displays a disrupted condition.

Another aspect of genetic mutations is the condition termed perdurance of wild type function (Garcia-Bellido and Merriam, 1971) and relates to the analysis of mosaics. In the normal mutant homozygote the mutant phenotype will be displaced. But if the homozygous tissue is induced by mitotic recombination from heterozygous cells (3.1.5), then the wild type product, synthesised in the mother heterozygous cell, may persist in clone cells resulting, and thus mask the expression of the mutant phenotype (Garcia-Bellido and Merriam, 1971).

4.2 Information derived from some useful mutations

In the natural condition, most somatic cells of organism possess the same genetic complement, and it is the selective transcription of particular loci which is responsible for the varied differentiation displayed by cells. It is not unreasonable to suspect that a single locus, or small number of loci, may be responsible for the differentiation of a particular cell, and as a result mutations may be expected which disrupt this differential process.

Some mutations cause the elimination, whether by non formation or exclusion from the developing system, of a particular cell type, and in the highly ordered system, such as in ommatidial patterning, elimination, particularly of receptor cell types, can be useful in decyphering the developmental process.

The sevenless mutant was isolated by Benzer (see Harris et al, 1976) and is characterised by the ommatidia possessing only seven, instead of the normal eight, receptor cells. Campos-Ortega et al, (1979), after a series of

experiments, were able to conclude that the locus relates only to the R_7 cell, and in the mutant condition this cell is absent. No evidence was found to suggest that the sevenless locus is involved with any cytodifferentiation process, the mutation prevents the R_7 cell from, either forming, or entering into the developing R cell clusters. It is known that the R_7 cell is produced in the terminal mitosis of the receptor cells. Analysis of the perdurance effect showed that the wild-type allele is required by dividing precursor cells to produce the R_7 cell, so indicating that the R_7 cell determination process operates before the cell itself is produced.

Ransom has isolated two mutations, both of which have a similar effect to sevenless (see Campos-Ortega, 1980). Unlike sevenless, however, these are recessive lethals and cannot be studied with such ease, the mutant cells only survive when present in a clone induced in a wild-type background. Both these mutations 1(2)ff225 and 1(2)ff40 lack two of the receptor cell group $R_{1,6} + 7$, and the former may lack all three. Again with these two, analysis of perdurance tentatively indicates that the wild-type gene is required, prior to their generative mitosis, to ensure the cells' presence (Campos-Ortega, 1980).

Another mutation isolated by Ransom, 1(2)ff10, (see Campos-Ortega, 1980) causes rhabdomeric formation beneath the basement membrane and non-formation of cone cells. Again this mutation can only be studied in mosaics. It is thought that the target cells of this locus are in fact the cone cells (Campos-Ortega, 1980), and the rhabdomeric disruption is a secondary effect, as is the disruption of other ommatidial elements. Here again it is found that if the wild-type gene is present,

0-48 hours before pupation, then development is normal. 1(2)ff10 is particularly interesting in that two other mutations - lz^{50e} and spa^{pol} produce a similar phenotype. The lz^{50e} locus, by perdurance analysis, appears to be important during the proliferative phase of the eye tissue i.e., the phenotype of the mutation cannot be uncovered when the homozygosity, is induced after the second instar. The spa mutation differs from the other two in that its mode of action is non cell autonomous (Campos-Ortega, 1980). Thus there are three loci which affect development in a similar manner but by different methods and so indicate complexity in the genetic control of development.

CHAPTER 2. MATERIALS AND METHODS

1. FLY STOCK MAINTENANCE

Flies for experimentation were reared in incubators at 25°C, a low temperature of 18°C being used for stock maintenance. Individual stocks were maintained in glass or plastic vials containing solid food (Table 2.1).

2. FIXATION METHODS

2.1 Fixation of head discs

The following is a modification of the methods of Campos-Ortega and Gateff (1976) and Perry (1968a).

Larvae of appropriate ages were immersed in cooled Drosophila Ringer solution (Table 2.2), the low temperature inhibiting movement and allowing easier manipulation. Using two pairs of forceps the mouth-parts and attached cephalic complex were dissected from each larva and dropped into cooled 2% glutaraldehyde solution (Table 2.3). The volume of fixative was then doubled by adding 2% osmium tetroxide (Table 2.4), effectively reducing the glutaraldehyde and osmium concentrations by half. The tissue was left in this mixture, over ice, for thirty minutes and then thoroughly washed in buffer (Table 2.5) before being placed in 2% osmium solution alone for two hours. After a further wash in buffer the tissue was dehydrated in a cooled, graded alcoholic series (Table 2.6). The absolute alcohol was replaced with propylene oxide (a far more effective solvent of the resin) and after thirty minutes and a change of solvent an equivalent volume of resin (Table 2.7) was added and the mixture shaken vigorously to facilitate the dissolution of the resin.

The dissolved resin was allowed a minimum of eight hours to infuse the tissue before being replaced by pure resin and after five hours the dissection of the material was then continued. Using a needle and forceps the cephalic complex was separated from the mouth parts and the head discs teased free from their contact with the brain hemispheres. The discs were now orientated as desired in fresh resin and polymerisation proceeded at 70°C for a minimum of 72 hours.

2.2 Fixation of adult eyes

Adults were anaesthetised with CO₂ and then decapitated using a razor blade. The head was then bisected leaving the two eyes separated and supported by head tissue. Fixation and embedding was performed as described for the head discs.

3. SECTIONING

Sectioning was performed on a Reichert-Jung Ultracut, a Reichert-Jung OMU3, or a Cambridge Huxley Microtome.

3.1 Light microscope sectioning

Sectioning was performed on glass knives cut from a LKB Knife Maker and the water bath was provided by an LKB Bath Mould. 0.75-1.75µm sections were cut and removed from the water bath in a fine platinum loop and transferred to a water drop on the surface of a slide upon a hot-plate.

3.2 E.M. sectioning

E.M. sections were cut on an Emscope 3mm diamond knife, but when thick sections were required from the same block a glass knife was used. Silver sections provided the optimum qualities between contrast and resolution and these were picked up on a variety of grids, the most commonly used being the Gilder G200 Hex.

3.2.1 Serial sectioning

Serial sectioning was performed so that information derived from examination of the series could be used to produce a three dimensional reconstruction of the retinal tissue. It proved essential to be able to identify, on each section examined, not only the same relative area of tissue but also the same individual cells. To establish with certainty the correlation between sections it was found that section should not be separated by more than four silver/gold sections. Progressive ribbons of five sections were picked up on grids, the central section being the one examined and the remainder used for references when necessary.

(a) Slot Grids and Coating

Grid bars cannot only upset the visual continuity of the section and hence prevent accurate location of the desired patch of tissue, but also tend to lie on or around the cells being followed. To prevent this, Gilder GS2x1 slot grids were used with an overlying Formvar film providing support for the sections.

Many coated grids were required and the following procedure was used to produce them. A 2% Formvar solution (Table 2.8) was placed in

a burette and a cleaned microscope slide was immersed. The liquid was drained away at a rate that would give the desired thickness of film. The film was then floated off the slide onto a surface of distilled water, underneath which a wire mesh carrying slot grids was positioned. The water was drained away and the film brought to rest, and allowed to dry, over the grids.

The thickness of film chosen was dependant upon the expected usage of the grid. A thicker film reduces resolution and a thinner one is more mechanically weak. Since up to 60 grids could be used in a series and the loss of one or more grids at any stage could destroy the series then the strength of the film was an important factor. Tests with various film revealed that a silver film was the minimum thickness that would provide sufficient durability without appreciably reducing resolution of the section. However, this thickness film tended to reduce the contrast in a section and these were accordingly cut as silver/gold - slightly thicker than would normally be used. To prevent breakage of the Formvar film in the electron beam, the specimen stage was cooled with liquid nitrogen, the filament current was kept as low as possible, and the accelerating voltage used was 40Kv (this also enhancing contrast).

4. STAINING

4.1 Staining of sections for light microscopy

Sections dried on a microscope slide were covered with toluidine blue solution (Table 2.9a), placed on a hot plate (80°C) for 30-60 seconds, washed with distilled water and a coverslip was mounted with DPX.

When autoradiographs were to be stained, the stain needed to pass through the overlying gelatinous emulsion without the solution dissolving the emulsion. A different toluidine blue solution was now used (Table 2.9b), the temperature reduced to 70°C and staining was performed a few seconds at a time.

4.2 Staining of sections for E.M.

Grids carrying dried sections were inverted onto a drop of Reynold's lead citrate (Table 2.10) surrounded by sodium hydroxide pellets for up to ten minutes in a humid atmosphere. The grids were then thoroughly washed with distilled water and dried.

5. MICROSCOPY

5.1 Light microscopy

Light microscope sections were examined using a Leitz Dialux 20 microscope.

5.2 Electron microscopy

Tissue was examined using a Phillips 301, and a Joel 100S, transmission electron microscope.

In examination of the serial sections, the magnifications used were standardised to ensure ease of interpretation of the information. Preliminary micrographs were taken from chosen patches of tissue from the central section of each grid at a magnification of 1300X. From this photographic series individual groups of cells were

chosen. The sections were re-examined and a magnification of 5900X was used to provide detailed information. When necessary information was found to be lacking between two photographs of the series, intermediate sections were now examined to provide the detail needed.

6. PHOTOGRAPHY

6.1 Light microscopy

Ilford FD4 35mm film was used for light microscopy. It was developed in Acutol Developer and fixed in Ilford Hypam.

6.2 E.M.

Negatives taken from the electron microscopes used Ilford Technical Film and Kodak 4463 Electron Image Film. They were developed in Ilford PQ Universal Developer and fixed in Ilford Hypam.

All printing was done on Ilford Multigrade Photographic Paper. Developing was with Ilford Multigrade Developer and fixation with Ilford Ilfospeed Paper Fixer.

7. AUTORADIOGRAPHY

7.1 Labelling of the tissue

7.1.1 In vitro labelling with ^3H thymidine

The cephalic complex and attached mouthparts of third instar larvae were dissected and dropped into 1ml of culture medium. Kuroda and Yamaguchi (1956) have shown that culturing of the eye disc is more successful when contact with the cephalic complex is maintained.

Labelling was performed following a similar procedure to that described by Campos-Ortega and Gateff (1976). The tissue was cultured for 30 minutes in medium containing 20µl of sterile aqueous 48 Ci/mmol methyl ^3H thymidine per ml. The tissue was then thoroughly washed with medium and then, was cultured in fresh medium for varying lengths of time. Following the procedure adopted by Campos-Ortega and Gateff (1976), Drosophila Ringer (Table 2.2) was the medium initially used. However, examination by the electron microscope of head discs cultured in Drosophila Ringer revealed that considerable tissue damage had occurred. The Drosophila Ringer was now replaced by Shields and Sang Medium* (Shields and Sang, 1977) supplemented with 2% foetal bovine serum. Examination of tissue cultured in this medium revealed no degeneration or damage - the tissue appeared normal. It is suspected that the retinal developmental process occurring during the third larval instar may be related to the increasing level of the insect moulting hormone B ecdysone (Martin Milner, private communication). Retinal differentiation begins relatively early in the third larval instar and hence it was inferred that if the hormone is in some way involved, then a low concentration is effective at this stage. Fearing that a too high level of the hormone may induce morphogenesis then a low level of approximately 0.002µg/ml was used - this being in the order of 1/50 of that needed to induce morphogenesis.

7.1.2 In vitro labelling with ^3H Uridine

Unlike thymidine, which is a specific precursor of DNA, uridine need not automatically be incorporated into RNA. Uridine can be con-

* Kindly supplied by Martin Milner

verted via deoxyuridine 5'-phosphate to thymidine 5' phosphate, a direct precursor of components of DNA. However, for uridine to be taken up into DNA reductive methylation at the 5' position must occur. Hence, the uridine used to label RNA synthesis is [5- ^3H] uridine, any reductive methylation at the 5' position results in elimination of ^3H from the molecule (see Fenner, et al, 1974).

The cephalic complexes were dissected from third instar larvae and dropped into Shields and Sang medium containing 20 μl of sterile aqueous 27Ci/mmol ^3H uridine per ml. After thirty minutes the tissue was washed thoroughly in medium and fixation begun immediately.

7.1.3 In vivo labelling with ^3H thymidine

Third instar larvae were injected with approximately 0.1 μl of sterile aqueous 48Ci/mmol ^3H thymidine, following Ready et al, (1976). Larvae were prepared for injection following the procedure described by Ursprung (1967). The larvae were firstly washed in tap water followed by 70% alcohol. They were dried with filter paper and then anaesthetised with ether. The larvae were then placed on a microscope slide and muscular relaxation induced by being covered by a drop of Drosophila Ringers. The larvae, now elongate, were dried with filter paper and arranged upon the microscope slide to allow rapid and orderly injection.

The injection apparatus consisted of a fine, glass micropipette connected by plastic tubing to a reservoir containing the injection fluid which was delivered to the needle by a small, hand operated, peristaltic pump. The epidermis of each larva was punctured by the

needle and by squeezing the plastic tubing between the nails of the thumb and index finger liquid was expelled from the needle. Before relieving the pressure on the plastic tubing the needle was withdrawn. By this method, the amount of liquid delivered to each larva was dependant upon the bore of the plastic tubing used. By using plastic tubing of bore 0.5mm the desired volume, of approximately 0.1 μ l, was injected.

After injection, larvae were left for fifteen minutes before being covered with Drosophila Ringer. They were then transferred to a dampened piece of filter paper and supplied with a yeast solution. After varying lengths of time, the cephalic complexes were dissected from the larvae and fixed - any larva not appearing healthy was discarded.

7.2 Coating and exposure of the sections

1 μ m and 1.5 μ m sections were cut and dried onto gelatinised slides. The slides were dipped in Ilford K5 Emulsion diluted by half with distilled water, dried, and sealed in boxes containing silica gel and stored at 4°C.

At intervals of three days a slide from each group of sections was removed from storage and its emulsion developed in ID19 (Table 2.11) for five minutes at 25°C. After a wash in distilled water, the emulsion was fixed in 25% sodium thiosulphate and then washed in distilled water and dried at room temperature. When the correct exposure time for each group of slides was ascertained the remainder were developed and fixed accordingly.

TABLE 2.1 FOOD RECIPE

104g	Maize
506g	Agar
94g	Granulated Sugar
19g	Dried Yeast
5g	Nipagine
5ml	Propionic Acid
1000ml	Water

TABLE 2.2 DROSOPHILA RINGER'S SOLUTION

7.5g	NaCl
0.35g	KCl
0.21g	CaCl ₂
1000ml	Distilled H ₂ O

TABLE 2.3 2% GLUTARALDEHYDE SOLUTION

4ml	25% Glutaraldehyde
46ml	0.1M Phosphate Buffer (Table 2.5)

TABLE 2.4 2% OSMIUM TETROXIDE SOLUTION

2g	OsO ₄ Crystals
50ml	0.1M Phosphate Buffer (Table 2.5)

TABLE 2.5 0.1M PHOSPHATE BUFFER

Solution A (0.2M)

27.8g	NaH ₂ PO ₄
1000ml	Distilled H ₂ O

Solution B (0.2M)

71.7g	Na ₂ HPO ₄ (12H ₂ O)
1000ml	Distilled H ₂ O

Solution C (0.2M)

28ml	Solution A
72ml	Solution B

Solution D (0.1M)

100ml	Solution C
100ml	Distilled H ₂ O

TABLE 2.6STRENGTH AND DURATION OF THE ALCOHOL SERIES

30%	-	10 Minutes
50%	-	"
70%	-	"
80%	-	"
90%	-	"
100%	-	2 x 15 Minutes

TABLE 2.7DURCAPAN* RESIN

50g	Resin (Polyepoxide)
50g	Hardener (Dodecanyl Succinic Anhydride)
1.75g	Accelerator (Tri-Dimethylamino-Methyl-Phenol)
0.75g	Plasticiser (Di-Butyl-Phthalate)

* Polaron Equipment Limited, Hertfordshire

TABLE 2.8FORMVAR SOLUTION

2g	Formvar Resin
100ml	Chloroform (Analar)

TABLE 2.9 TOLUIDINE BLUE SOLUTION

- (a) 1g Toluidine Blue
 100ml 1% Borax Solution
- (b) 0.25g Toluidine Blue
 100ml Distilled H₂O
 Bring to pH9 with 0.1M NaOH

TABLE 2.10 REYNOLDS LEAD CITRATE

Solution A

- 2.66g Lead Nitrate
 30ml Distilled H₂O

Solution B

- 3.52g Sodium Citrate
 30ml Distilled H₂O

Solution C

- 30ml Solution A
 30ml Solution B
 Leave 30 Minutes
 16ml 1M NaOH
 24ml Distilled H₂O

TABLE 2.11 ID19

1.1g	Metol
36g	Sodium Sulphite (Anhydrous)
4.6g	Hydroquinone
24g	Sodium Carbonate (Anhydrous)
2g	Potassium Bromide
500ml	Distilled H ₂ O

CHAPTER 3. THE STRUCTURE OF THE DEVELOPING OMMATIDIAL BUNDLES

1. INTRODUCTION

The retina of Drosophila is an assembly of readily identifiable cells arranged in a precise and repeated pattern and accordingly is particularly suited for the analysis of the developmental processes that generates it.

Studies using mutations and agents that interfere with normal development reveal that the third larval instar is a period of particular importance in retinal development (see van Breugel et al, 1975). The head disc of Drosophila is peculiar in that differentiation begins, and is readily identifiable, during the third larval instar - cells of other imaginal discs do not display overt differentiation until the pupal stage has begun.

Although a considerable amount is known about the effects of interfering with third larval instar retinal development, by comparison only little is understood of the processes that actually occur during that period. The major work upon the eye of Drosophila that explains third larval instar development was performed by Ready et al, (1976). A detailed explanation of this work is given in Chapter 1. Briefly, this research revealed that undifferentiated eye tissue undergoes mitosis, then experiences the constriction of the morpho-genetic furrow (MF), after which preclusters of five receptor cells become evident, a second division now occurs generating cells from which the remaining three receptor (R) cells are taken and incorporated into the precluster forming the mature eight cell ommatidial bundles.

It is known that the cells of the precluster are the cells $R_{2,3,4,5 + 8}$ and the receptor cells derived from the second mitotic wave are the cells $R_{1,6 + 7}$.

With the present state of knowledge a variety of developmental models can be proposed to explain the processes occurring posterior to the MF. The models vary according to the questions which ask whether cellular interaction induces cellular determination or whether cellular interaction occurs because cellular determination is established. Much of the confusion could be assuaged by answering the following:-

1. The five cells of the pre-cluster are determined to form the cells $R_{2,3,4,5 + 8}$ but are any of them already individually committed to form a particular R cell of that group?
2. Are the three R cells, generated by the second division, to any extent determined before being taken up into the cluster?

A valuable technique adopted by the embryologist is the comparison of aberrant with normal development. Retinal differentiation is first established during the third larval instar and as a result it is not surprising that many failures in eye development, exhibited by the action of various mutations and experimental treatments, are initiated at this stage. However, an attempt by the present author to establish, by electron microscopic investigation, the cellular difference between the presumptive retinal tissue of the third larval instar of various mutations with that of the wild type proved fruitless primarily due to the confusion that arises when cell identification is attempted. Although at certain developmental stages and depths in the prospective eye field eight cell

clusters of receptor cells are easily distinguishable, at other stages and depths, resolution into cone, pigment and receptor cells is not possible. Thus, it was decided that a detailed electron microscopical analysis of the retinal tissue posterior to the MF would be performed - firstly, to produce an atlas of the developing eye field, so allowing a simple and rapid method for identification of cells in the region of the mature eight cell ommatidial bundles, and secondly, to attempt to establish the process of eight cell bundle formation in a hope of being able to answer, to a certain extent, the questions relating to the interaction/determination models.

2. TECHNICAL AND ANALYTICAL METHODS ADOPTED

2.1 The Plane of Sectioning

Because the process of development occurring in the third larval instar eye tissue displays a posterior to anterior progression, late instars were taken to ensure that a complete range of the developmental stages were present in the head disc. The specimens were serially sectioned and examined using the electron microscope as described in Chapter 2. This procedure is in itself very time consuming and was complicated further by the physical structure of the head disc. The head disc when viewed from a distal aspect can be considered as shown in Fig. 3.1. However, the median and lateral 'wings' of the eye region in reality bend and curve under the proximal side of the disc (Fig. 3.2). Again in the antero-posterior plane the disc is also curved (Fig. 3.3). This situation is complicated further as the larvae approach the prepupal period - contraction of the peripodial membrane begins to pull the presumptive eye region towards that of the anterior so shifting the posterior of the disc above the plane it was previously lying in (Milner et al, in proof). These problems of curvature, coupled with the relatively small size of the head disc itself, can result in only a small area of the tissue examined being sectioned in the plane required. As a result many discs needed to be sectioned and examined before the full range of developmental situations could be appreciated.

The plane of sectioning desired was that perpendicular to the proximo-distal axis of the ommatidial bundles (Fig. 3.4). The propinquity of the actual plane of cut to that desired, could be established, for each

patch of tissue under scrutiny, by examining the sections taken from the proximal region of the material. Here bundles of axons projecting from the presumptive ommatidial receptor cells are present. The axons are long, thin, cylindrical structures and a section through each bundle deviating appreciably from the plane perpendicular to the proximo-distal axis would show it to have elliptical rather than circular characteristics (Fig. 3.5). Accordingly, before an area of tissue was examined in detail it was established that the tissue was being examined in a plane sufficiently close to that desired.

2.2 Defining the cells

Ready et al, (1976) have shown that in the late third larval instar, at the posterior of the presumptive eye field, bundles of eight prospective receptor cells are evident. However, as previously indicated, the distinction of these eight cells can only be established at particular stages and positions in the posterior region of the eye field.

The technique adopted by the present author of using serial sectioning allowed the individual cells to be followed along their entire length and they could be identified at any level. Although receptor cells could often be identified by their inclusion into an ommatidial bundle, occasionally cells were of ambiguous identity. This problem of cell identity was resolved by defining the receptor cells as those bearing, proximally, an axonal projection. Accordingly, a serially sectioned ommatidial bundle was first examined proximally, the eight axonal projections identified, and systematically traced back through the preceeding sections to the distal tips of the cells.

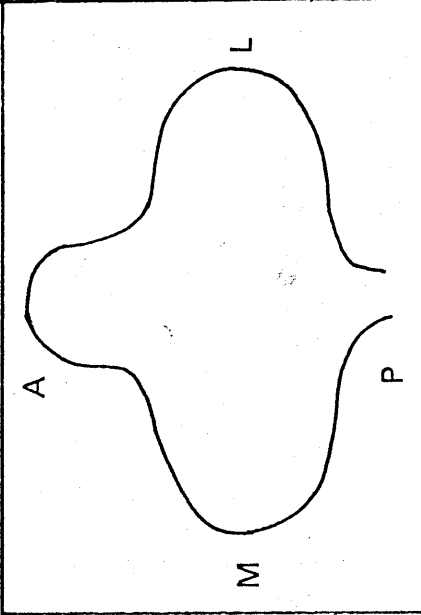


Fig. 3.1 The head disc as it appears from a distal aspect.
A: anterior; L: lateral; M: medial; P: posterior. Right eye.

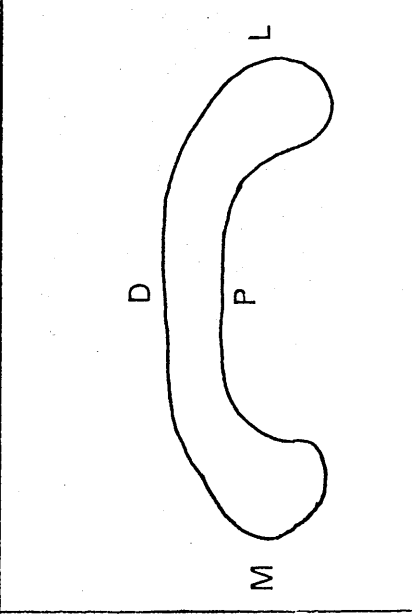


Fig. 3.2 The head disc as it appears in transverse section.
D: dorsal; L: lateral; M: medial; P: proximal

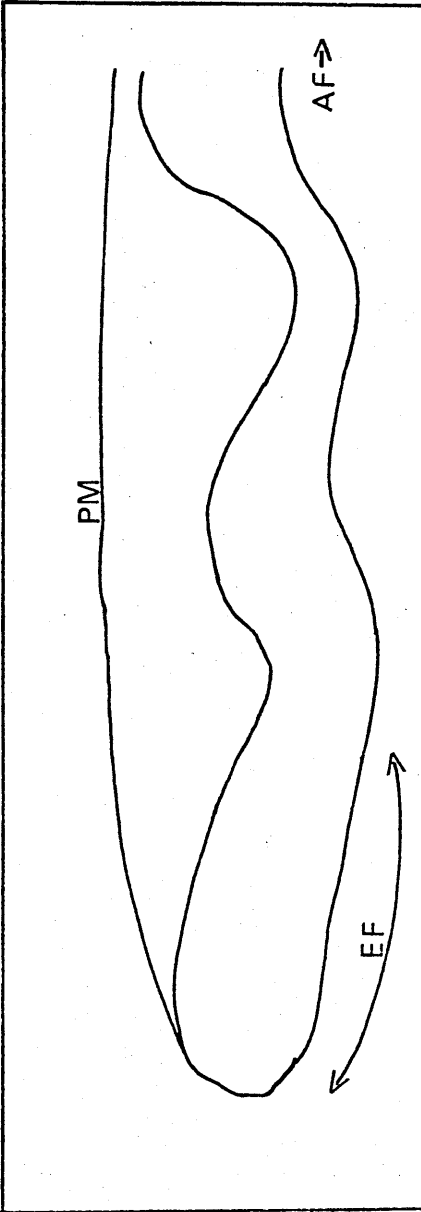


Fig. 3.3 The head disc as it appears in longitudinal section.
AF: antennal field; EF: eye field; PM: peripodial membrane

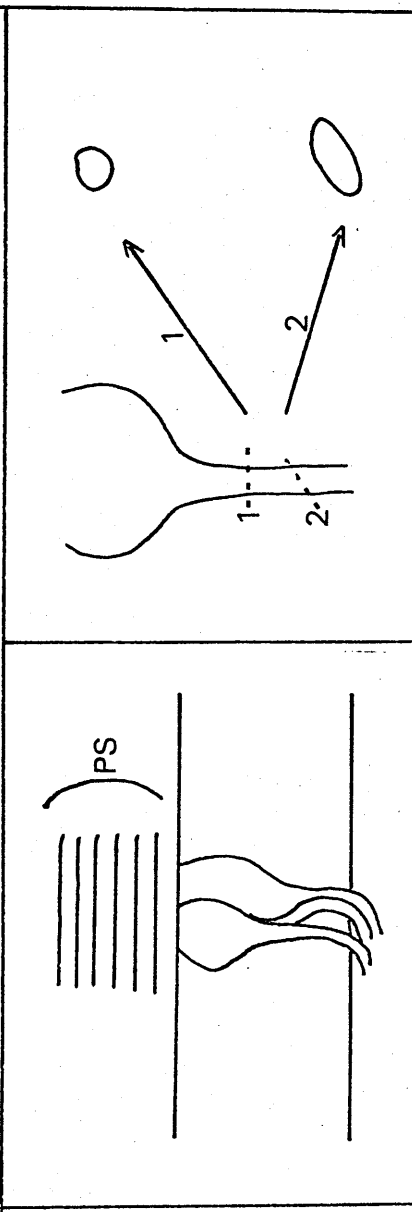


Fig. 3.4 Diagram showing the desired plane of sectioning (PS)

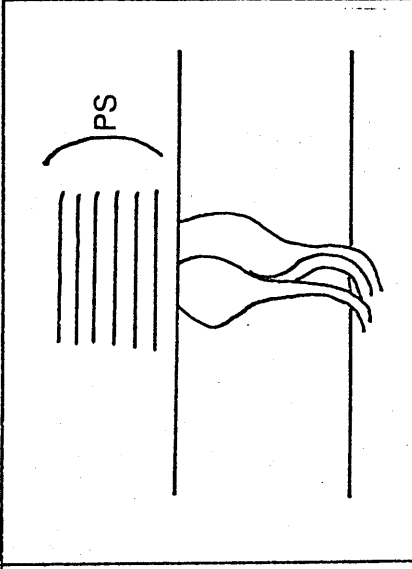


Fig. 3.5 Sections through axonal projections deviating from the desired plane of sectioning would show it to have elliptical (2) rather than circular (1) characteristics

Having established a method for distinguishing receptor from non-receptor cell, the identity of the individual cells in the grouping now arose. The adult ommatidia and mature (see 3.1) third larval instar ommatidial groupings bear very similar axonal bundles and hence the identity of the receptor cells within the groupings was inferred from the positioning of their projections within the axonal bundles. The central constituent of the adult axonal bundle is the projection from R_8 and accordingly the central axon of the third larval instar bundle was assigned R_8 identity. The other cells were identified by similar special relationships e.g. the row $R_1R_2R_3$ is found to the anterior of R_8 . Ready et al, (1976) also adopted this method for establishing cell identity and found that about the line of pattern inversion LPI (Chapter 1, 3.1.5) the pattern inversion was consistent with the assumed cellular identity. Further evidence of the accuracy of this method has come from light microscope autoradiography where it has been shown that cells labelled in the second mitotic wave occupy clustering positions consistent with their assumed identity (Ready, private communication). Confirmation of the validity of these assumptions accrued from the investigations described in this thesis where it was found that during the later stages of the third larval instar, cells moved in a manner consistent with that to produce the adult cellular arrangement and also examination of the mutation sevenless revealed that the cell assigned to be R_7 was in fact the one displaying aberrant behaviour.

3. THE STRUCTURE OF THE MATURE OMMATIDIAL BUNDLES

The experimental technique used in this analysis revealed a progression of retinal developmental stages, the extent of which was dependent upon the extent of posterior displacement from the MF. Initially two distinct developmental stages were recognised; the mature, which was defined as an ommatidial bundle containing eight cells each bearing proximally a constituent of an unequivocal axonal bundle, and the immature, which were characterised by failing to comply with this criterion. Although a complete series of developmental stages has now been appreciated, this was only achieved by first analysing and understanding the mature situation and then utilising this knowledge to explain that of the immature. In order to aid description of the developmental series the stages will be dealt with in the order that they were originally established.

In the mature ommatidial bundles, the nuclei, and hence the cell bodies of the receptor cells are found clustered in the distal half of the retinal tissue, and three developmental stages have been defined by the number of non-receptor cell nuclei also found in this distal clustering.

The development of the retinal tissue during the third larval instar appears to be continually progressive and it is not wished to imply that the stages described are developmentally more important than their intermediate forms, rather they were chosen by their ease of recognition.

3.1 The Stages of Mature Ommatidial Bundling

STAGE I

The Stage I ommatidial bundle is the first mature grouping to be established and is distinguished from its later counterparts by the lack of any non-receptor cell nuclei in the distal region. The receptor cell body clustering occupies a distal to central location and their emanating axonal projections pass through the proximal region, where the non-receptor cell-bodies are found, to the developing optic stalk. On reaching the optic stalk the axons change direction and run, in the axis perpendicular to the ommatidial bundle, posteriorly under the more mature stages. From the lumen surface to the developing optic stalk is approximately 40-45 μ m, this length was found to vary slightly when different head discs were examined. With the exception of the rearrangement of the receptor cell-bodies, the rising to the distal surface of certain non-receptor cell nuclei, and the shortening of the overall ommatidial proximo-distal length, this essential arrangement of the retinal tissue does not change appreciably during later third larval instar development.

The Stage I cluster contains a central receptor cell (R_8) surrounded by seven others and throughout the entire length of the developing receptor cell bundle the central cell bears no contact with any cell other than the surrounding seven receptor cells which, with the exception of certain situations in the axonal bundle, contact it at all levels.

Just below the lumen surface, extending proximally for about 4 μ m, a large number of distinct zonulae adherens were evident. In this region most cells are only represented by small distal projections to the lumen

surface and although cell identification at this level is difficult it can be said that each receptor cell is joined to its every neighbour by an adherens type junction and it is strongly suspected that all cells are connected by these junctions (Fig. 3.6a).

During Stage I, and successive third larval instar stages, the retinal tissue retains the immature characteristic of being a monolayer of columnar epithelium cells. Although cell bodies are found at different proximo-distal locations every cell extends from the lumen surface to the most proximal levels.

The line of pattern inversion (LPI), which runs equatorially along the antero-posterior axis of the disc, is the boundary either side of which the clustering pattern shows mirror-image inversion. Thus the pattern inversion takes place along the medio-lateral axis and as a result a particular cell positioned laterally in a cluster in the ventral part of the eye will be positioned medially in the dorsal part. Hence, the terms equatorial and polar will be used with reference to the direction of the LPI and prospective poles of the eye respectively.

The receptor cell-body clustering at this stage produces a square arrangement and the organisation of the cells are consistently regularly organised along the entire proximo-distal axis (Fig. 3.6). The anterior flank of the central receptor cell (R_8) is bordered by cells R_1 and R_2 (R_1 being placed equatorially) and cells R_6 and R_5 occupy a similar posterior location. Polarity is given to the arrangement by two cells (R_3 and R_4) being found at the polar border of the central cell and the equatorial position being occupied by the remaining R_7 cell (Fig. 3.6).

This polarity of the cell patterning allows a ready method for cell identification i.e. cell R_7 is easily distinguishable as it is placed opposite two cells (no other cell is found with this characteristic) and having established the location of R_7 in the grouping, cell R_1 is identified as being found anterior to it and the remaining eccentric receptor cells are arranged around the central cell in the order that their cell identification numbers imply.

With the exception of the axonal bundle where a certain amount of 'jostling' for position can go on, the equatorio-polar axis of the Stage I bundling is a line about which the clustering arrangement is symmetrical (Fig. 3.6b). This symmetry results from the cells R_1, R_2, R_3 being arranged to the anterior of the line of symmetry as the cells R_6, R_5, R_4 , respectively, are to the posterior, the central and R_7 cells both being bisected by the line (Fig. 3.6b). Cells R_1 and R_6 , R_2 and R_5 , and R_3 and R_4 show paired arrangement, each pair of cells being organised about the central cell, one to the anterior and the other in a similar manner to the posterior (Figs. 3.6; 3.7,* a,b,c). Six of the receptor cell-bodies are found considerably more distally placed than the remaining two. Cells of the pair R_3 and R_4 are found at the polar positioning of the group and their cell-bodies are found most distal. The most equatorially positioned pair is that of cells R_1 and R_6 , their nuclei being located slightly more proximally than those of R_3 and R_4 and more

* The cellular arrangement when displayed by computer graphics is data derived from the entire series of sections - for explanation the reader is referred to the Appendix.

FIGURE 3.6

Sections taken from a Stage I mature ommatidial bundle. All receptor cells are numbered.

Fig. 3.6a x 10,000. 4 μ below distal surface. Note the adherens type junctions inter-relating the receptor cells. Nuclei of R₃ and R₄ are evident.

Fig. 3.6b x 10,000. 8 μ below distal surface. The nuclei of the pair R₁ and R₆ are now encountered. The equatorio-polar line of symmetry existing within the bundle is indicated.

Fig. 3.6c x 10,000. 15 μ below distal surface. The nuclei of R₃ and R₄ are no longer present and those of the pair R₂ and R₅, and R₈ are now evident.

Fig. 3.6d x 10,000. 25 μ below distal surface. The most proximally placed receptor cell nucleus (R₇) is now found. Note the two cone cell nuclei.

Fig. 3.6e x 10,000. 30 μ below distal surface. The nuclei of cell R₇ persists to a level where other receptor cells are reducing to their axonal elements. C: cone cells.

Fig. 3.6f x 10,000. 35 μ below distal surface. The axonal bundle of eight elements which projects into the optic stalk.

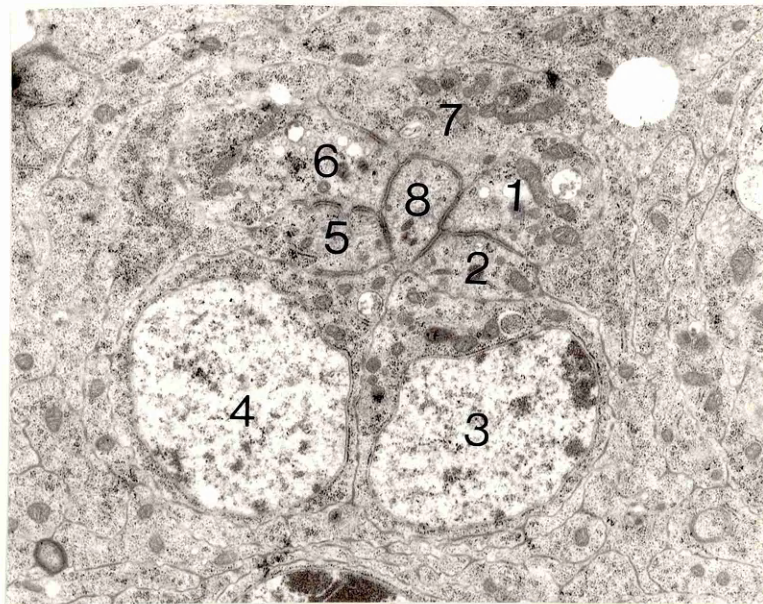


Fig. 3.6a

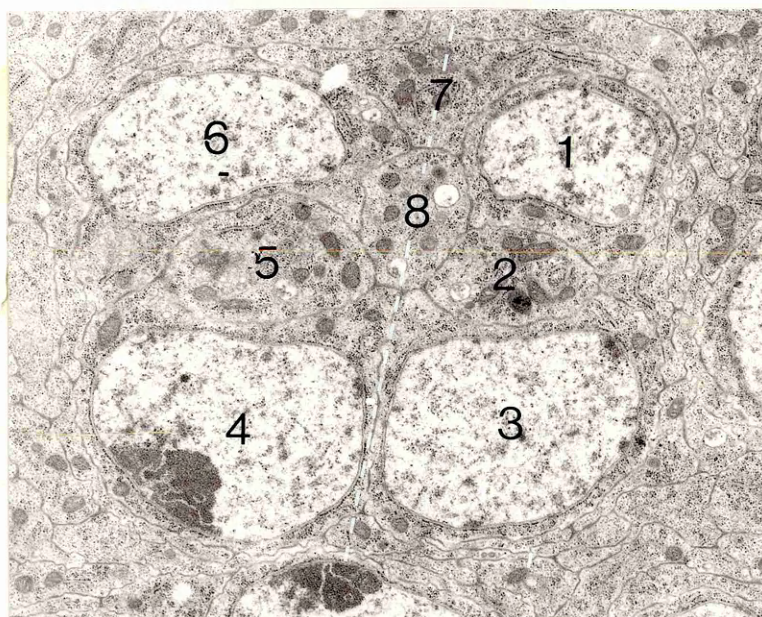


Fig. 3.6b

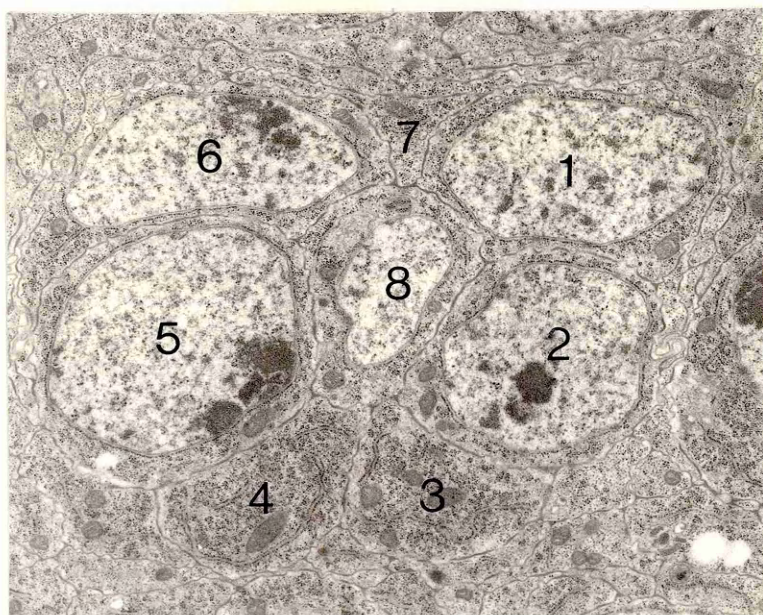


Fig. 3.6c

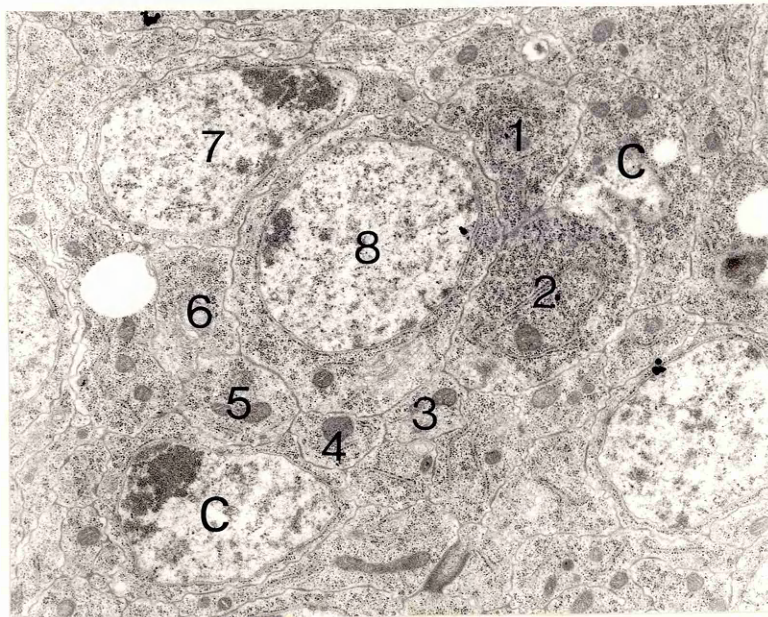


Fig. 3.6d

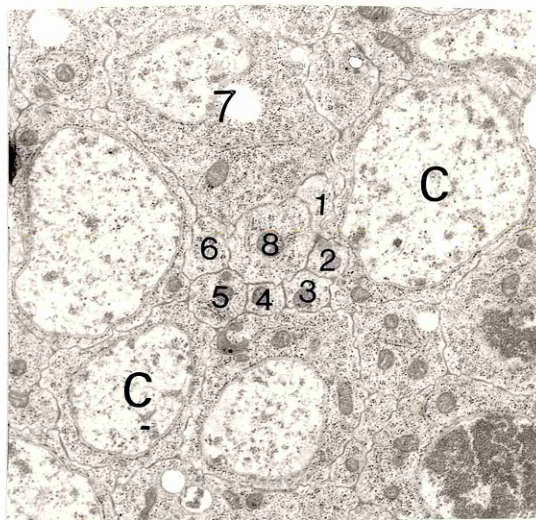


Fig. 3.6e

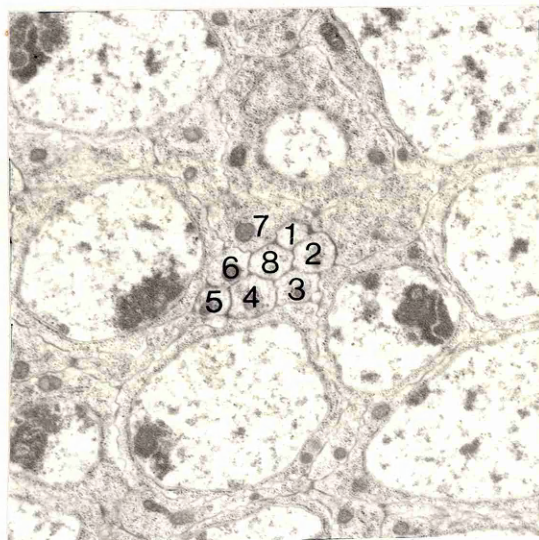


Fig. 3.6f

A Note on the Computer Graphic Print-outs

A feature of the reconstruction program allowed the ommatidial bundles to be rotated in the X/Y plane. In the legend to each print-out presented an angle is given which indicates the position from which the cells are being viewed.

The topographical viewing position indicated by the angles is displayed in the diagram below. Note the mirror-image arrangement either side of the line of pattern inversion (dotted line). 0° indicates that the cells are being viewed from a polar (PL) aspect, 90° an anterior (A) one, 180° equatorial (E), and 270° indicates a posterior (P) view point. Angles that are intermediate to those given above indicate an appropriately intermediate position.

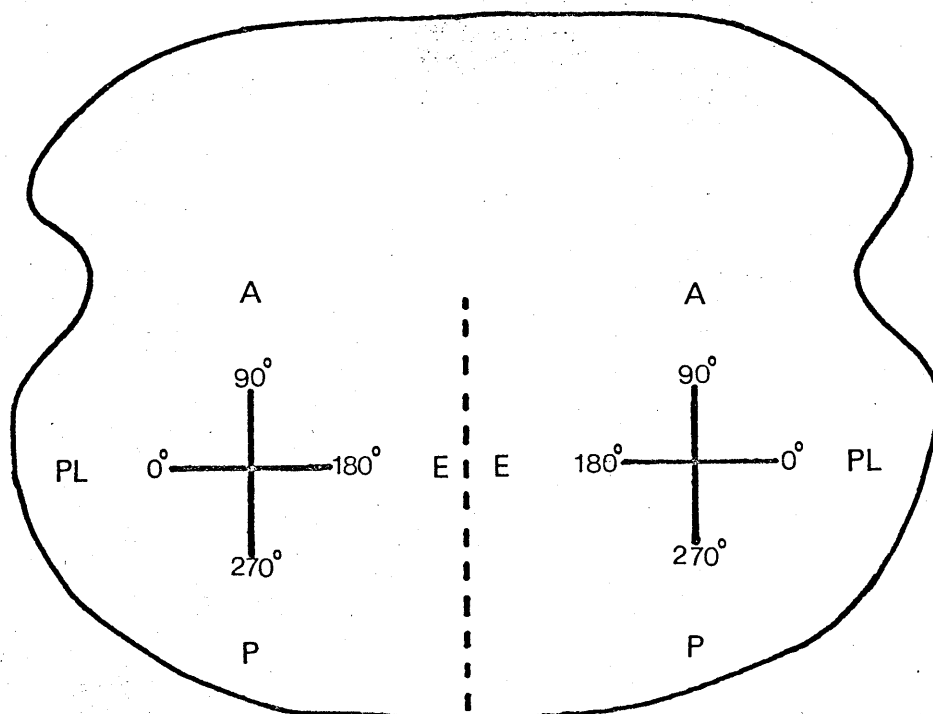


Diagram explaining directions of reference given on the computer print-outs.

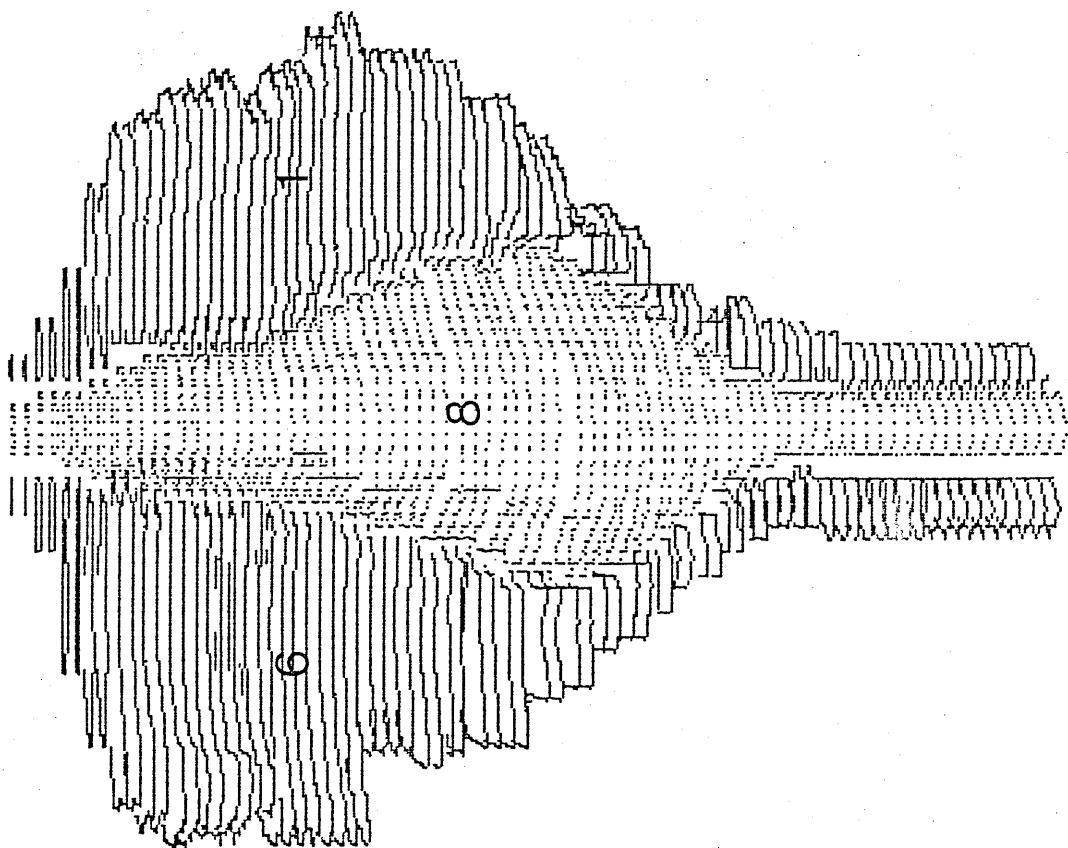


Fig. 3.7a Stage I. 0°. Posterior is to the left.
The paired arrangement of cells 6 and 1
about 8.

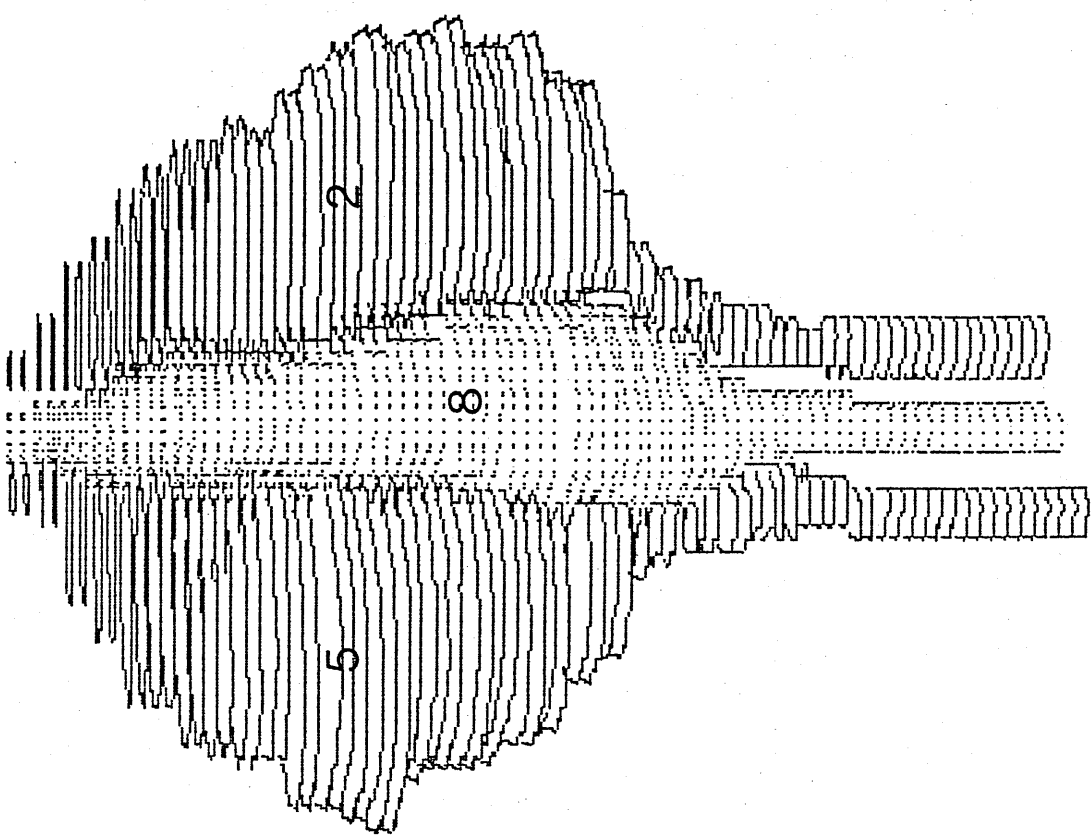


Fig. 3.7b Stage I. 0°. Posterior is to the left.
The paired arrangement of cells 5 and
2 about 8.

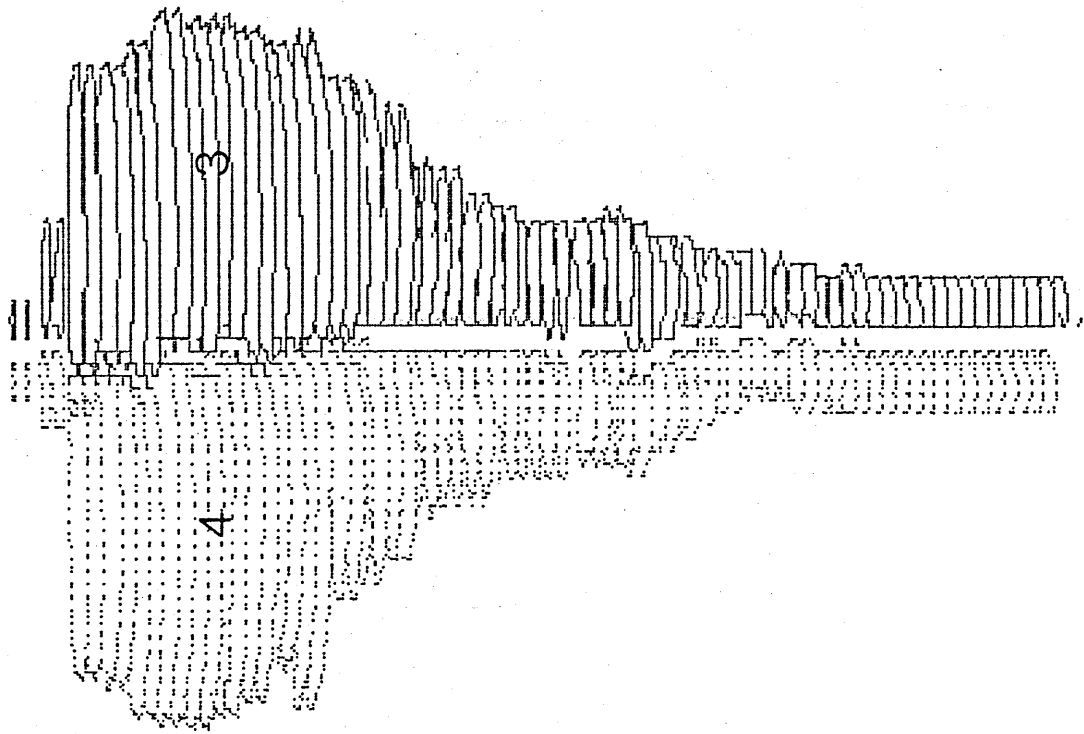


Fig. 3.7c Stage I. 0°. Posterior is to the left.
The paired arrangement of cells 3 and 4.

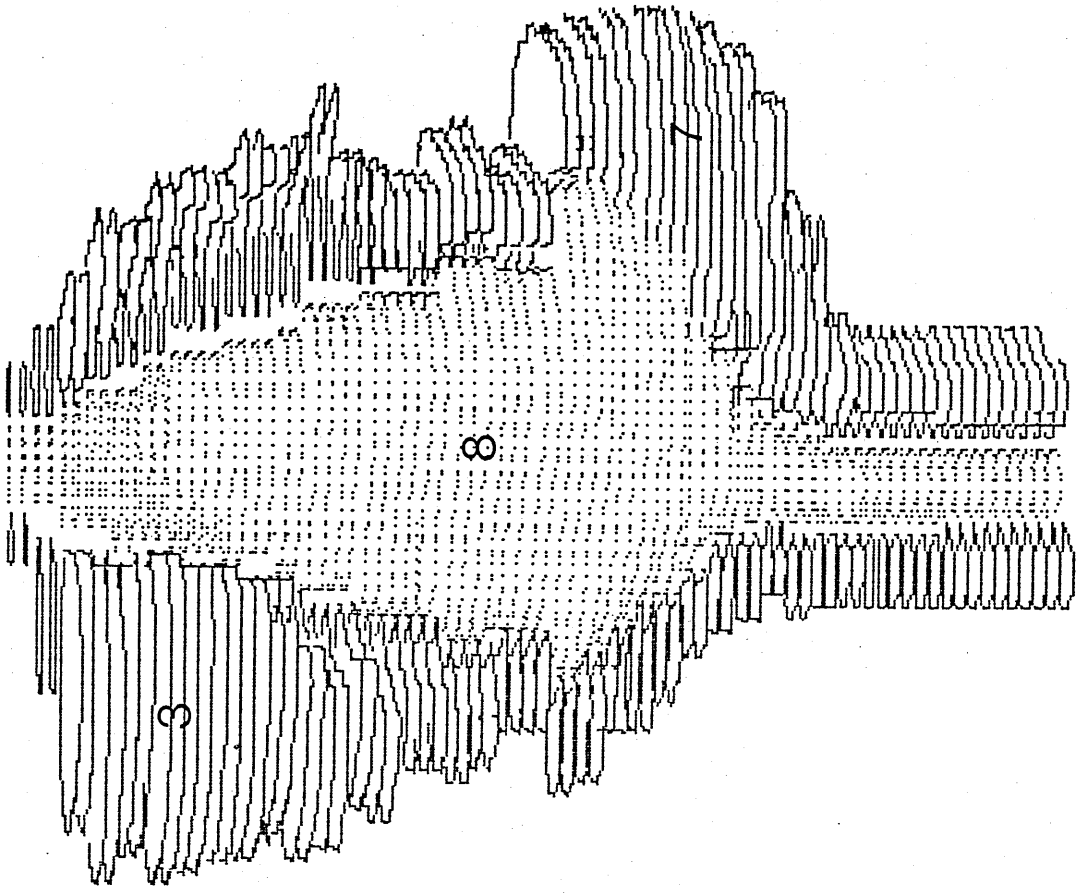


Fig. 3.7d Stage I. 90°. Polar is to the left.
The arrangement of cells 3 and 7 about 8.

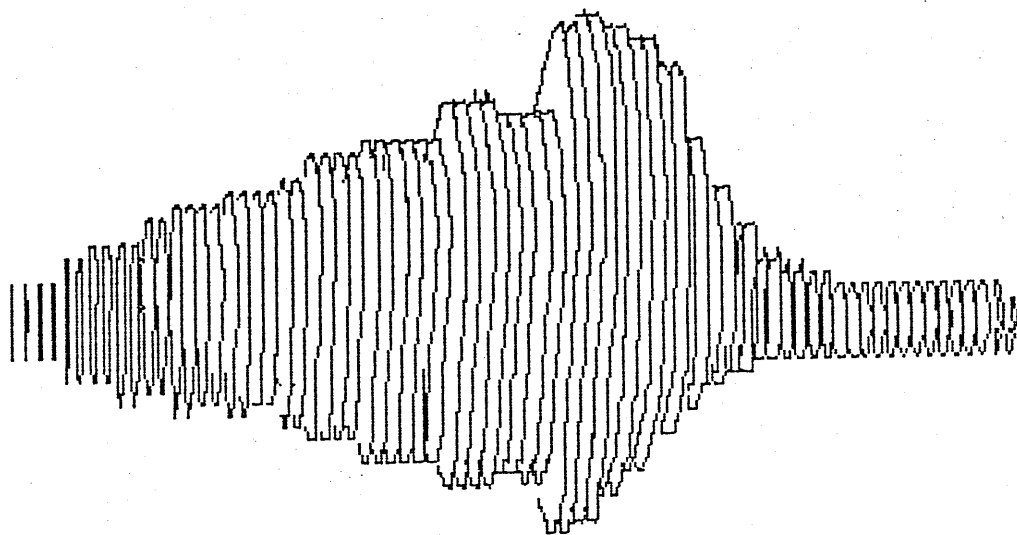


Fig. 3.7e Stage I 90°. Polar is to the left. Cell 8.

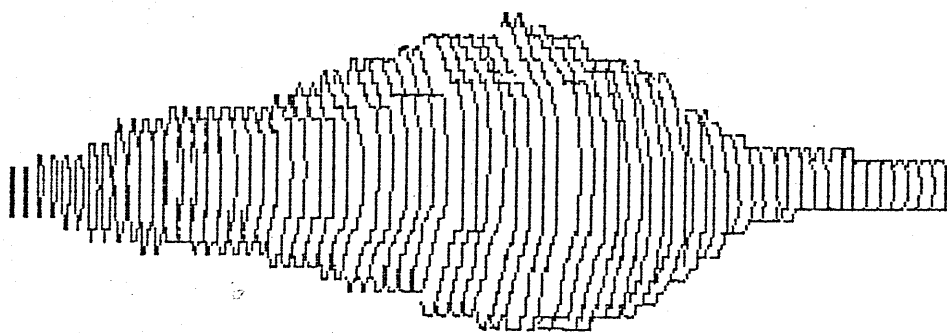


Fig. 3.7e Stage I. 0°. Posterior is to the left. Cell 8.

distally than those of R_2 and R_5 (Fig. 3.7 a,b,c). This packing arrangement, with the cell-bodies of the three pairs being placed at different levels, allows all six cell bodies to contact the central cell. The nucleus of the central cell (R_8), in relation to the nuclei of the six paired cells, is more proximally placed, and it occupies a central proximo-distal location (Figs. 3.6; 3.7). The cell-body of cell R_7 is found equatorially placed about the central cell, it begins at the lower levels of the central cell-body and is so proximally placed that its nucleus is still evident in sections taken from a depth where the other cells are only represented by their developing axonal projections (Figs. 3.6e; 3.7d).

As previously explained, at this stage all non-receptor cell nuclei are found in proximal locations, none are found in the region of the receptor cell-body clustering. During Stage I, as is the case with the later stages, all cells are of similar appearance, no cytoplasmic differentiation is evident and as a result the cells can only be separated into receptor cells (defined by their axonal projections) and non-receptor cells. However, two non-receptor cell nuclei can be identified, in a relatively distal location, at the level of the lower cell-body clustering (Fig. 3.6d). The significance of the positioning of these cells will be discussed in the description of Stage II.

STAGE II

The Stage II ommatidial bundling is characterised by the presence of two non-receptor cell nuclei in the distal clustering of the receptor cell-bodies (Fig. 3.8a). The overall length of the ommatidial bundle,

the distance from the lumen surface to the level where the axonal bundle joins the developing optic stalk, has been reduced by about 5μ from that found in Stage I to $35-40\mu$.

At the lumen surface the distal tips of the cells still show a similar arrangement of the adherens type junction found in Stage I.

The cell-bodies of all receptor cells have been shortened in the proximo-distal plane and the cell-body clustering has been shifted distally resulting in the axonal projections emanating from a more distal location (Fig. 3.9). The essentially square arrangement of the clustering found in Stage I has, as a result of the movement of the cell body of R_7 , now taken on, in the equatorio-polar axis, a more elongate appearance (Fig. 3.8).

The nuclei of cells R_3 and R_4 still occupy the most distal positioning in the grouping, but the nucleus of cell R_7 , found in Stage I as the most proximally placed, has risen up the clustering to the level of the cell bodies of R_3 and R_4 (Fig. 3.8a), but being a larger nucleus it extends some $2-3\mu$ below the nuclei of R_3 and R_4 . Hence, a section through the cluster at a level of approximately $5-10\mu$ below the lumen surface reveals the nuclei of cells R_3 , R_4 and R_7 , the remaining receptor cells (R_1 , R_2 , R_5 , R_6 and R_8) are represented by distal projections which are arranged in a characteristic 'bow-tie' shape and are bordered by the two non-receptor cell nuclei (Fig. 3.8a).

A movement by the R_4 cell-body away from contact with the distal part of the central cell is apparent at this stage (Fig. 3.8a), this being the initiation of the considerable displacement of this cell body from the distal clustering which becomes manifest in Stage III. With the exception of this slight displacement by cell R_4 , the equatorio-polar line of symmetry, and cell pairing, described in Stage I, persists (Fig. 3.9a,b,c).

The nuclei of the eccentric receptor cells in Stage I were arranged in four levels; distally to proximally they were R_3 and R_4 , R_1 and R_6 , R_2 and R_5 , and R_7 . However, in the Stage II clustering these nuclei are positioned at only two levels - R_3 , R_4 and R_7 being found most distally and R_1 , R_2 , R_6 and R_5 being found slightly more proximally. Although the nucleus of the cell R_7 is found close to the lumen surface, its axonal projection emanates from a considerably more proximal position than its counterparts with distally placed nuclei. This is thought to result from the recent migration of the R_7 cell nucleus from the proximal location i.e. the cell body of cell R_7 is found extended along the proximo-distal axis of the cell body bundling because the large nucleus has recently passed through it (Fig. 3.9d).

In the Stage I clustering each receptor cell, at any level in the bundling, was always found at the same relative position on the central cell border and never lost contact with its neighbouring receptor cells, and consequently a non-receptor never gained contact with the central cell. However, in the Stage II ommatidial bundle, although all receptor cell nuclei have shifted distally, the central cell-body is relatively more proximally placed and as a result the eccentric receptor cells (with

the exception of R_7 - see above) at the proximal levels of the cell body of R_8 are represented by their axonal projections, which because of their small size, do not occupy the entire central cell border and consequently non-receptor cells gain contact with the central cell (Fig. 3.8d,e).

The axonal processes from cells R_3 and R_4 and the lower cell-body of cell R_7 maintain the same position about the central cell body as their cell bodies do about its distal projection i.e. the cell R_7 always occupies the equatorial location and cells R_3 and R_4 are positioned at the polar border (Fig. 3.8). This is not the case with the other eccentric receptor cells. The axonal processes of cells R_1 and R_2 , found anteriorly, and R_6 and R_5 , found posteriorly, bend, with a polar displacement, around the central cell body as they are traced proximally, R_1 initially separating from R_2 and R_6 and R_5 likewise (Fig. 3.8d), but at the proximal level of the central cell body they come to lie either side (R_1 and R_2 anteriorly, R_6 and R_5 posteriorly) of the paired axonal processes of cells R_3 and R_4 resulting in a row of six projections which form a 'cap' for the polar border of the central cell body (Fig. 3.8e).

The two non-receptor cell nuclei found in the distal clustering are located either side of the 'bow-tie' arrangement (Fig. 3.8a) and lie above the cell bodies of R_1 and R_2 anteriorly and R_6 and R_5 posteriorly. These two cells are those identified in Stage I as being more distally placed than their counterparts, and examination of intermediate stages has revealed that they rise up the ommatidial body to the distal position they come to occupy.

FIGURE 3.8

Sections taken from a Stage II mature ommatidial bundle. All receptor cells are numbered.

Fig. 3.8a x 10,000. 5 μ m below distal surface. Two cone cell nuclei (C) are now distally placed. The nuclei of cells R₃, R₄ and R₇ are present, note the 'bow tie' arrangement formed by the distal projections of the remaining receptor cells.

Fig. 3.8b x 10,000. 8 μ m below distal surface. The nuclei of the pairs R₁ and R₆, and R₂ and R₅ are now encountered. Note the nucleus of R₇ persists more proximally than R₃ and R₄.

Fig. 3.8c x 10,000. 15 μ m below distal surface. The nucleus of R₈ is the most proximally placed.

Fig. 3.8d x 10,000. 18 μ m below distal surface. Note how non-receptor cells gain contact with R₈.

Fig. 3.8e x 10,000. 22 μ m below distal surface. Note how the axonal projections from cells R₁₋₆ form a polar cap about the central cell.

Fig. 3.8f x 10,000. 30 μ m below distal surface. The bundle of eight axons which project into the optic stalk.

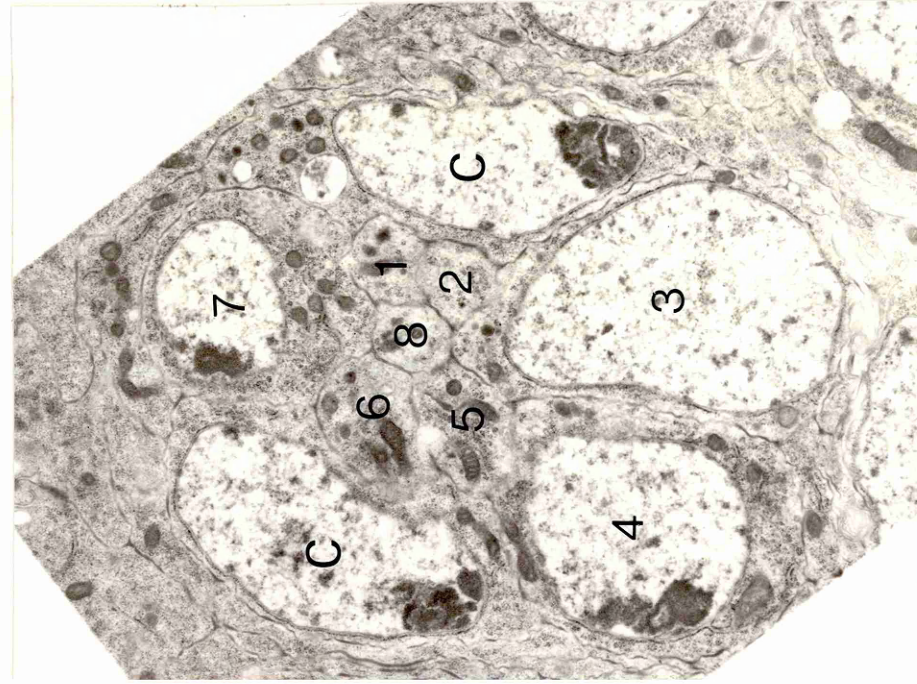


Fig. 3.8a

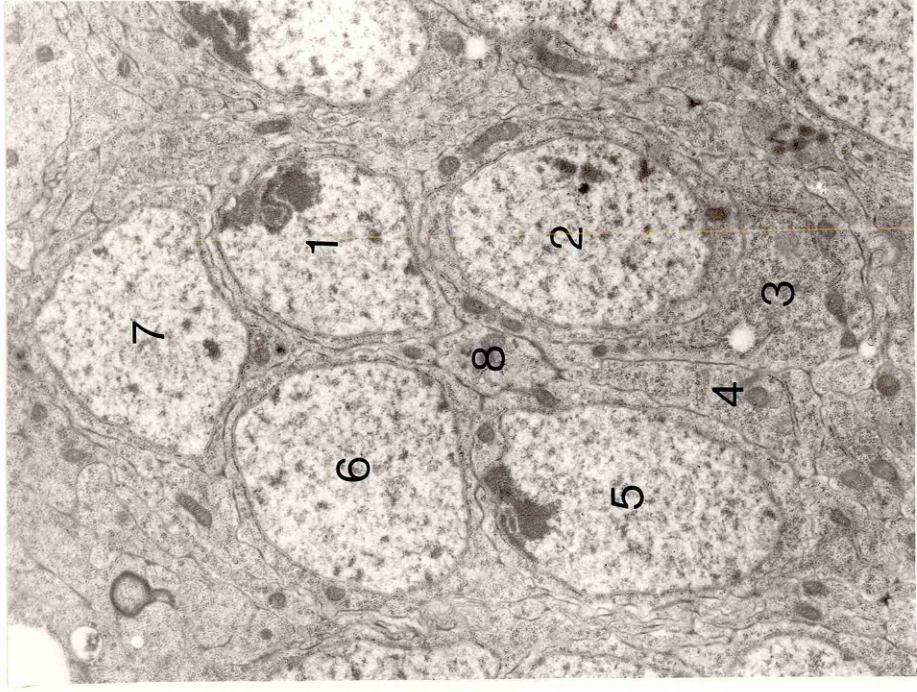


Fig. 3.8b

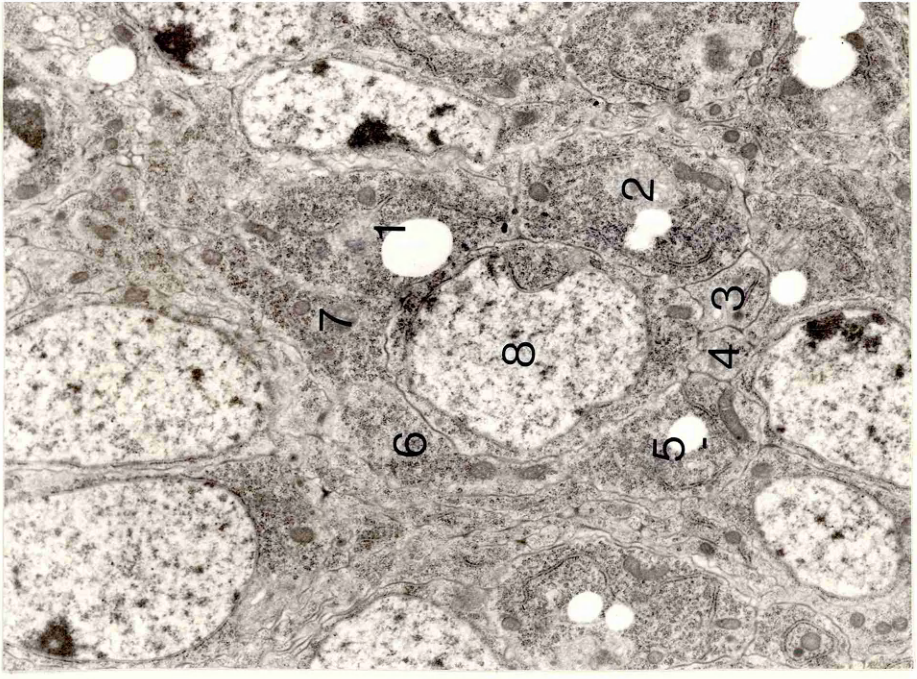


Fig. 3.8c

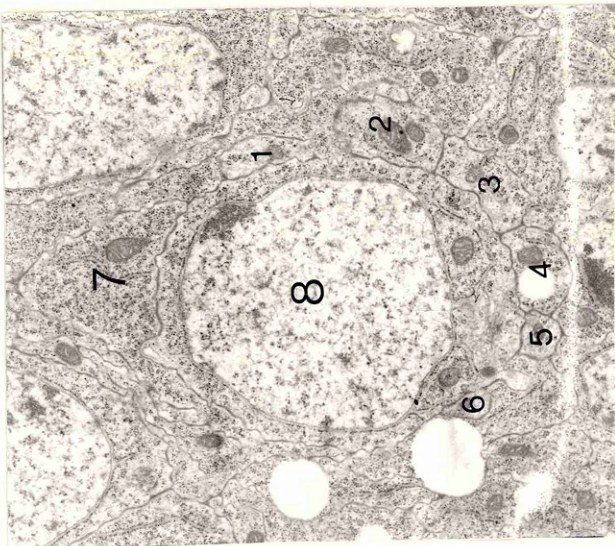


Fig. 3.8d

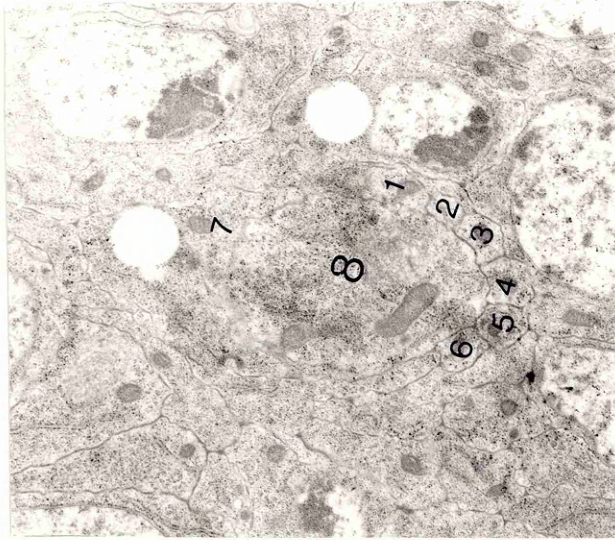


Fig. 3.8e

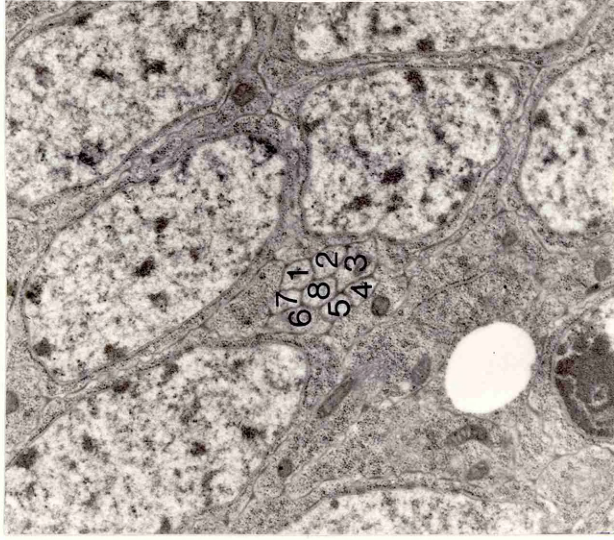


Fig. 3.8f

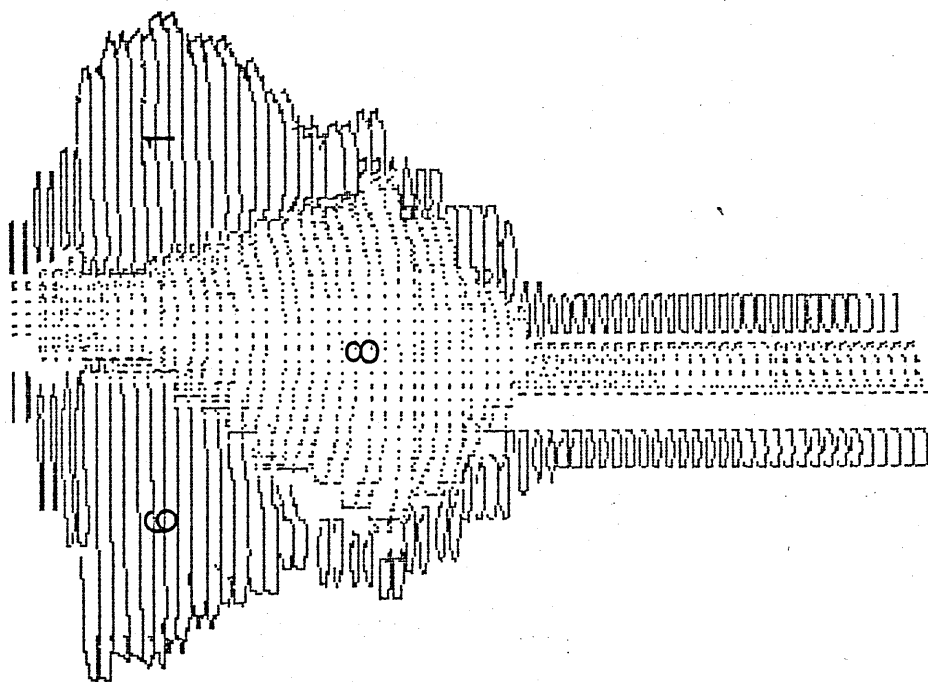


Fig. 3.9a Stage II 0°. Posterior is to the left. The paired arrangement of cells 6 and 1 about 8.

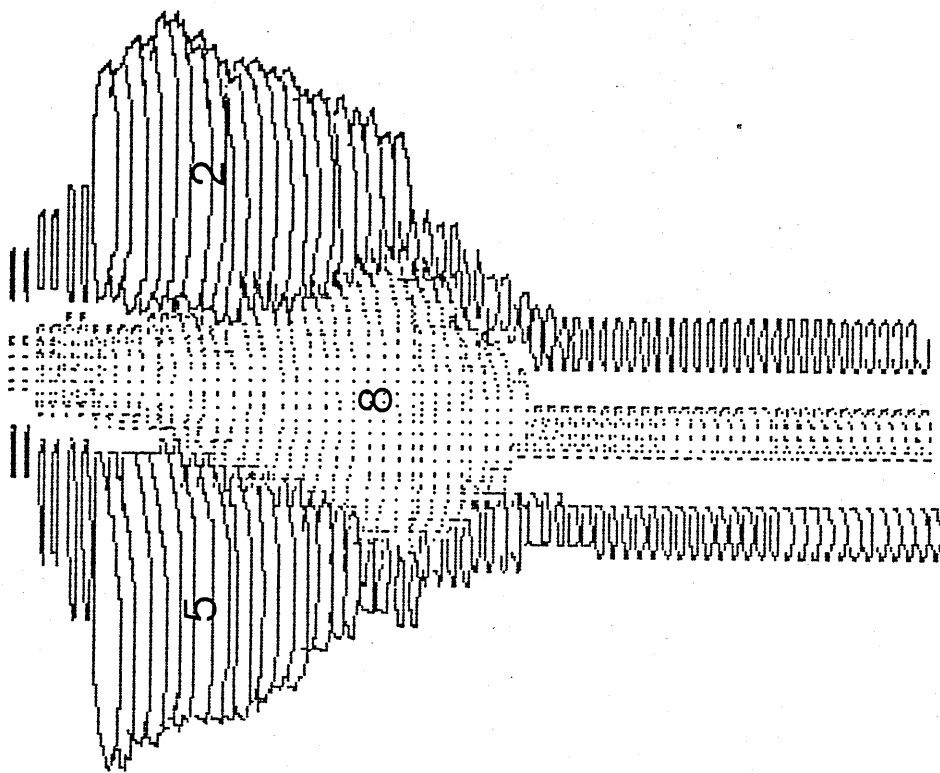


Fig. 3.9b Stage II 0°. Posterior is to the left. The paired arrangement of cells 5 and 2 about 8.

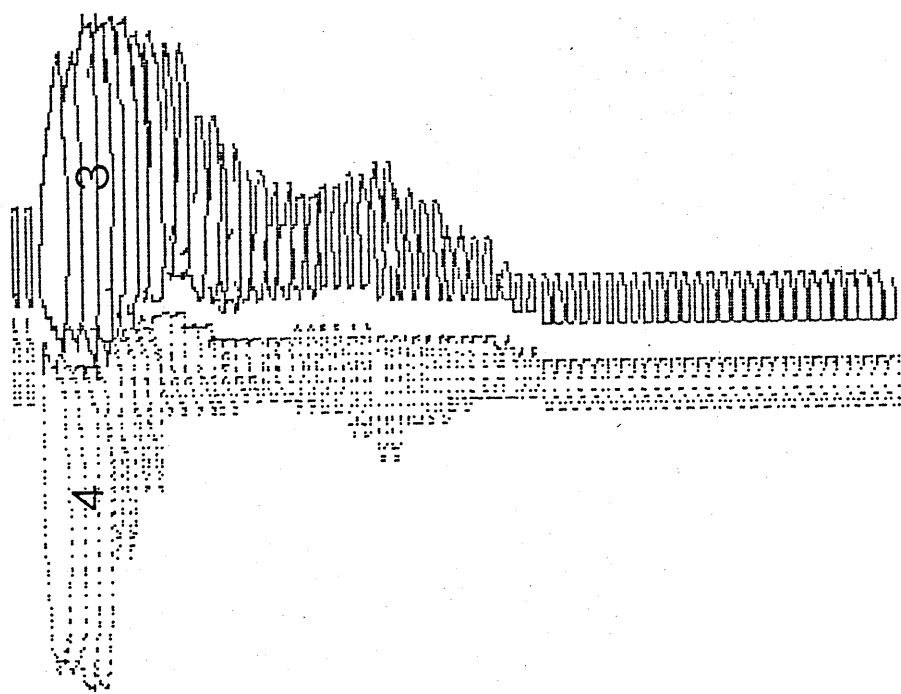


Fig. 3.9c Stage II 0°. Posterior is to the left. The paired arrangement of cells 3 and 4.

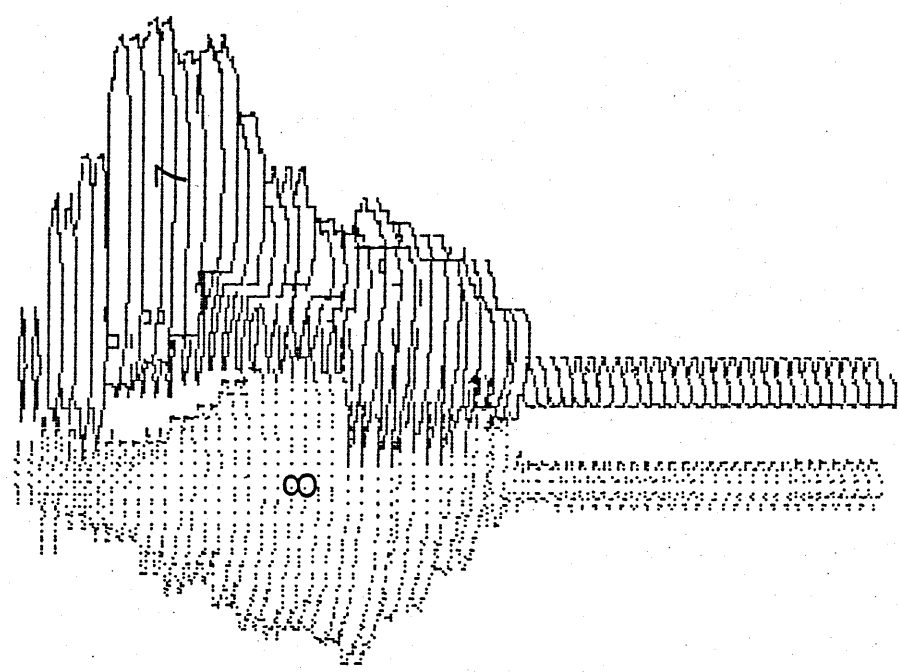


Fig. 3.9d Stage II 135°. Polar is to the left. Cells 8 and 7.

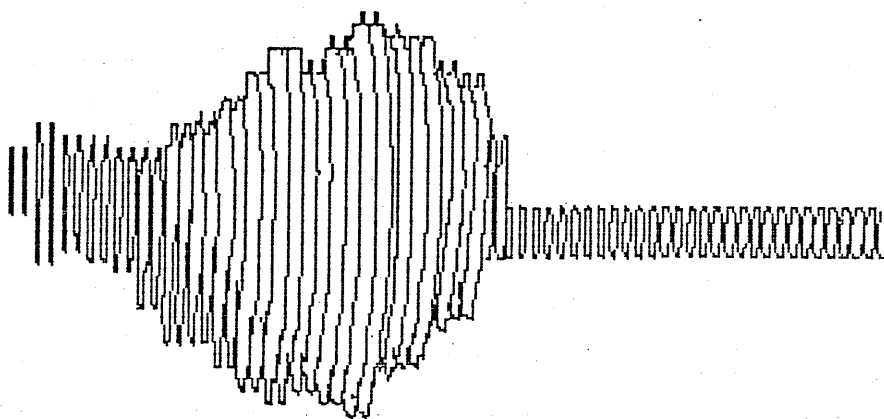


Fig. 3.9e Stage II 90° Polar
is to the left.
Cell 8.

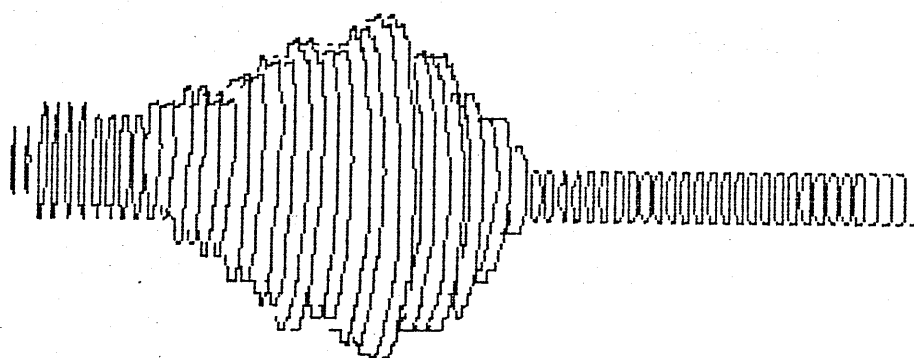


Fig. 3.9e Stage II 0°. Posterior
is to the left. Cell 8.

Cell division is evident in the region of the Stage II clustering and it is assumed that this is the limited mitotic activity occurring after the two major waves of division have passed (see Chapter 1, 3.1.4).

STAGE III

The Stage III ommatidial bundling is the final cellular arrangement achieved by the developing retinal tissue before pupariation and is characterised by the presence of four non-receptor cell nuclei about the distal tips of the receptor cells (Fig. 3.10a). Again, just below the lumen surface, the adherens type cell junctions were found in similar arrangement to that in Stage I. The reduction in overall ommatidial length, detected between Stages I and II, has continued, Stage III being approximately $10\mu\text{m}$ shorter than Stage I in the order of $30\text{--}35\mu\text{m}$.

All receptor cell nuclei have moved proximally from their locations in Stage II resulting in eight receptor cell distal projections being evident in the most distal $4\text{--}5\mu\text{m}$ of the tissue (Fig. 3.10a) and the axonal processes, emanating from a proximal region, are, coupled with the overall ommatidial length reduction, consequently reduced in length (Fig. 3.11).

The equatorio-polar line of symmetry, existing in the clustering during Stages I and II, no longer persists, the receptor cell bundling now having distinctly different characteristics. The distal projection of the cell R_4 is posteriorly displaced from its bundled seven counterparts (being occluded by two of the non-receptor cells (Fig. 3.10a))

and although, at a slightly deeper level, it joins the clustering, to gain contact with the central cell it skirts the extremity of cell R_5 before coming to occupy its axonal position between cells R_3 and R_5 , this only being achieved at the most proximal region of the cell body clustering (Fig. 3.11b). The central cell body has shown a considerable anterior and proximal shift from Stage II and the essential circular characteristic displayed in this earlier stage has been replaced by an antero-posterior elongation (Figs. 3.10; 3.11a). The displacement displayed by the central cell body now results in its distal and axonal processes emanating posteriorly and centrally respectively (Fig. 3.11a). The eccentric characteristics of the remaining cells are now lost, they now cluster around the posterior, equatorial and polar positions of the central cell body, the anterior half of which juts out of the bundling and is no longer involved in receptor cell contact (Figs. 3.10d,e; 3.11c).

At the distal regions of the clustering the 'bow-tie' arrangement of the distal projections of the cells $R_{1,2,6,5}$, and 8 , although slightly more proximally placed, is as found in Stage II (Fig. 3.10b). As progressively more proximal sections are examined it becomes apparent that the paired arrangement of the cells described in Stages I and II no longer exists. Cell R_4 having moved out from the distal clustering now no longer shows paired arrangement with cell R_3 , which alone now occupies the polar location in the distal grouping, opposite cell R_7 (Fig. 3.10a,b), all three of these cells, as in Stage II, having the most distally placed nuclei (Fig. 3.10b). The cells R_3 and R_7 show mirror-image arrangement about the central cell and hence these two are now showing a paired

arrangement (Fig. 3.11d), but examination of sections slightly proximal to the axonal bundle reveals that cell R_3 is reduced to its axonal component a little more distally than cell R_7 (Fig. 3.10e).

The cell bodies of R_1 and R_2 (R_2 being the more distally placed of the two) are found positioned at the anterior side of the distal projection of R_8 (Fig. 3.10c) at a more proximal level than those of cells $R_{3,4}$ and 7 . However, the anterior displacement of the central cell body results in the separation of the lower cell bodies, and axonal processes of the cells R_1 and R_2 to its equatorial and polar borders respectively (Figs. 3.10d,e; 3.11c).

The distal projections of cells R_6 and R_5 are arranged on the posterior border of the central projection (Fig. 3.10a,b,c) with R_6 being found equatorially, and their nuclei have shown a considerable proximal displacement from their location in Stage II, now lying at the level of the central cell-body. Owing to the anterior displacement of the central cell body (Fig. 3.11a) the axonal bundle is found more anteriorly placed, in the proximo-distal axis, than the corresponding distal projection and consequently all receptor cells, to an extent dependant upon their location within the cluster, at the central level of the bundling show a bend resulting from an anterior shift. Because the anterior surface of the central cell-body is not involved in receptor cell contact the cells tend to occupy a different location about the distal central projection than they do about the central cell body. Cells R_6 and R_5 , however, are always found positioned at the posterior border of R_8 (Fig. 3.10) and with their nuclei being found at the same level they show mirror-image arrangement about the antero-

FIGURE 3.10

Sections taken from a Stage III mature ommatidial bundle. All receptor cells are numbered.

Fig. 3.10a x 10,000. 5 μ m below distal surface. The distal projections of the receptor are surrounded by the cone cell-bodies (C). Note the displacement of R_4 .

Fig. 3.10b x 10,000. 8 μ m below distal surface. R_4 has now gained contact with the receptor cell grouping. Note the 'bow tie' arrangement of $R_{1,2,5,6 + 8}$.

Fig. 3.10c x 10,000. 12 μ m below distal surface. Note how R_4 is excluded from contact with R_8 .

Fig. 3.10d x 10,000. 15 μ m below distal surface. R_4 begins to skirt R_5 . Note how the cell body of R_8 pushes out between R_1 and R_2 .

Fig. 3.10e x 10,000. 22 μ m below distal surface. R_4 has now gained contact with R_8 in its expected location. Note that the anterior of R_8 is not involved in receptor cell contact.

Fig. 3.10f x 10,000. 28 μ m below distal surface. The eight axons which project into the optic stalk.

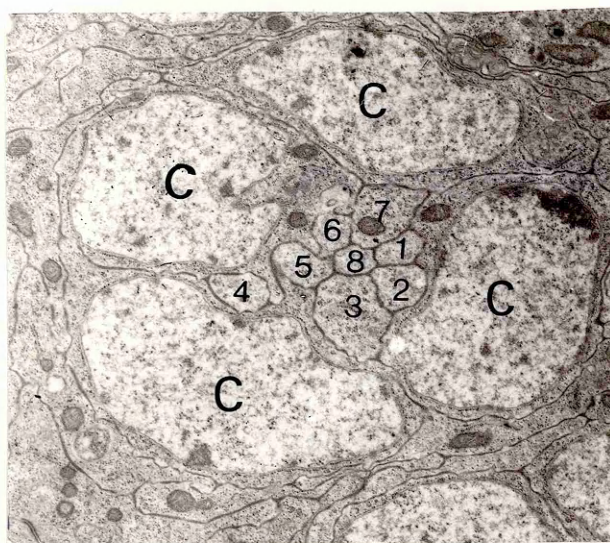


Fig. 3.10a

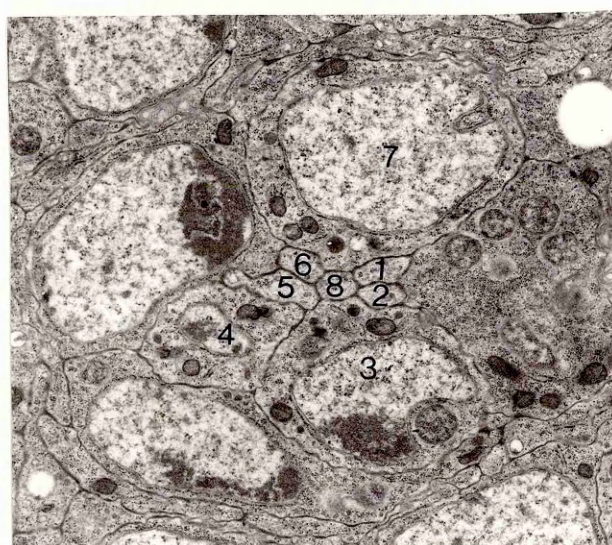


Fig. 3.10b

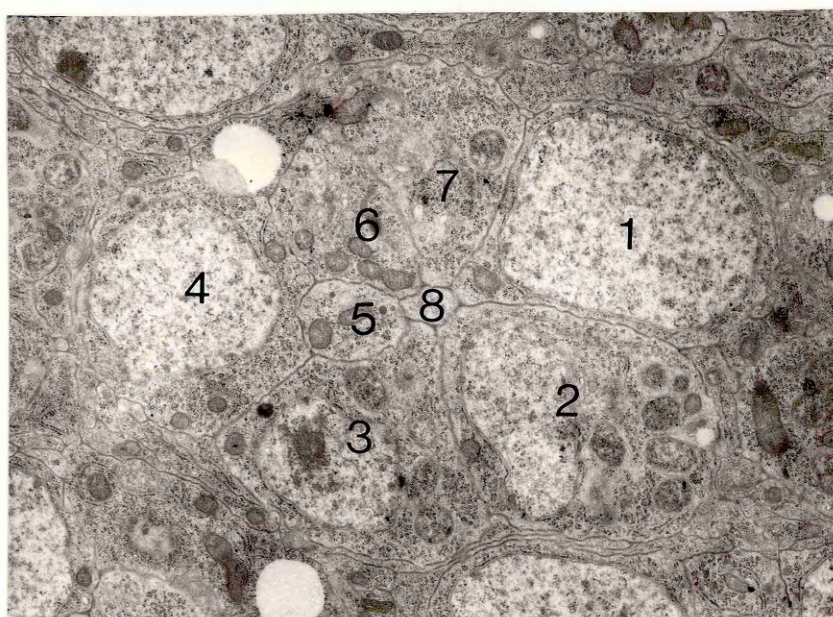


Fig. 3.10c

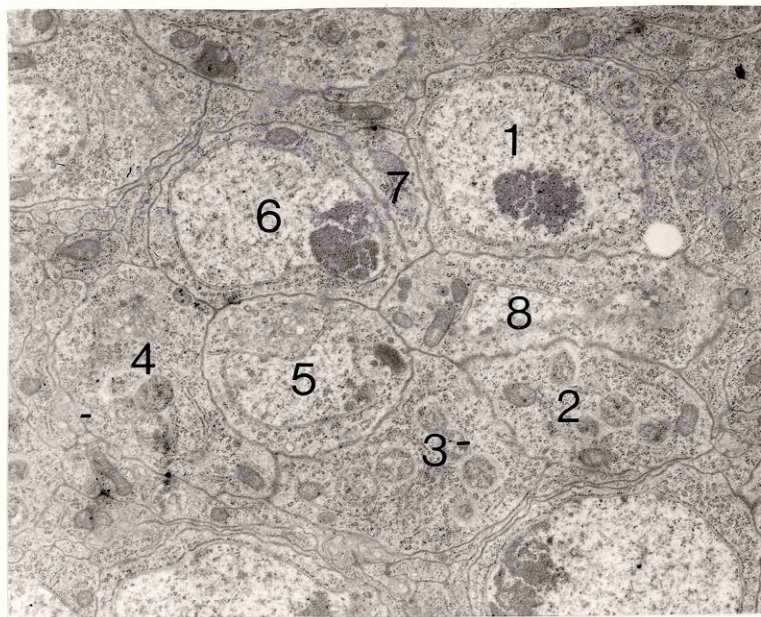


Fig. 3.10d

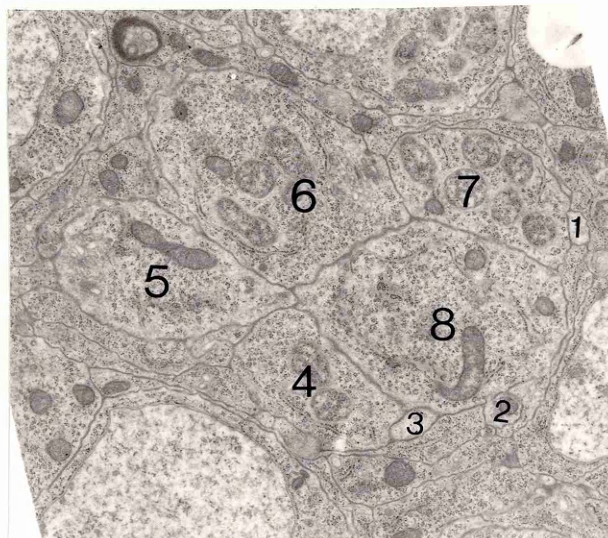


Fig. 3.10e

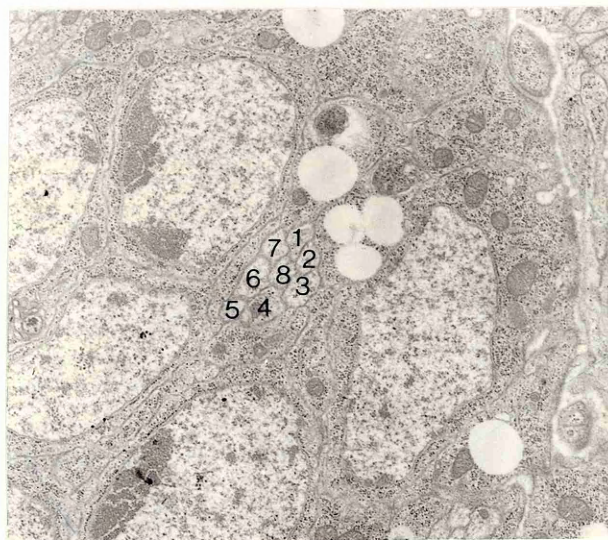


Fig. 3.10f

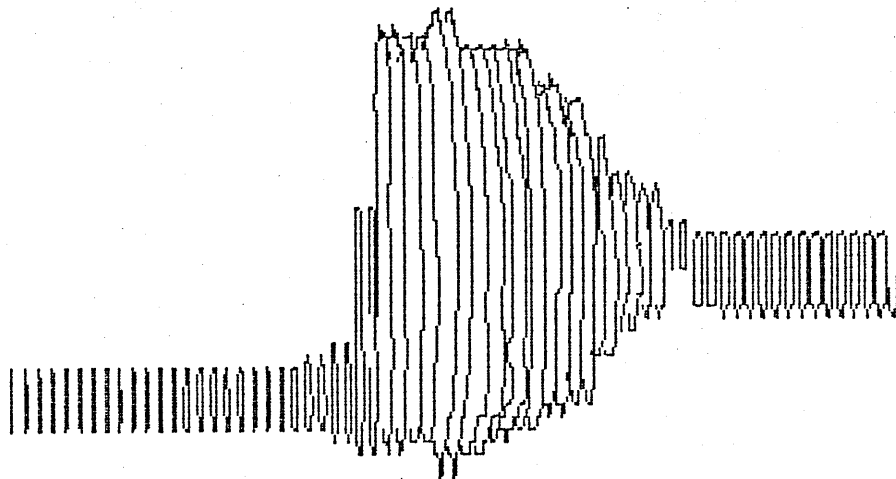


Fig. 3.11a Stage III 0°
Posterior is to
the left.
Cell 8.

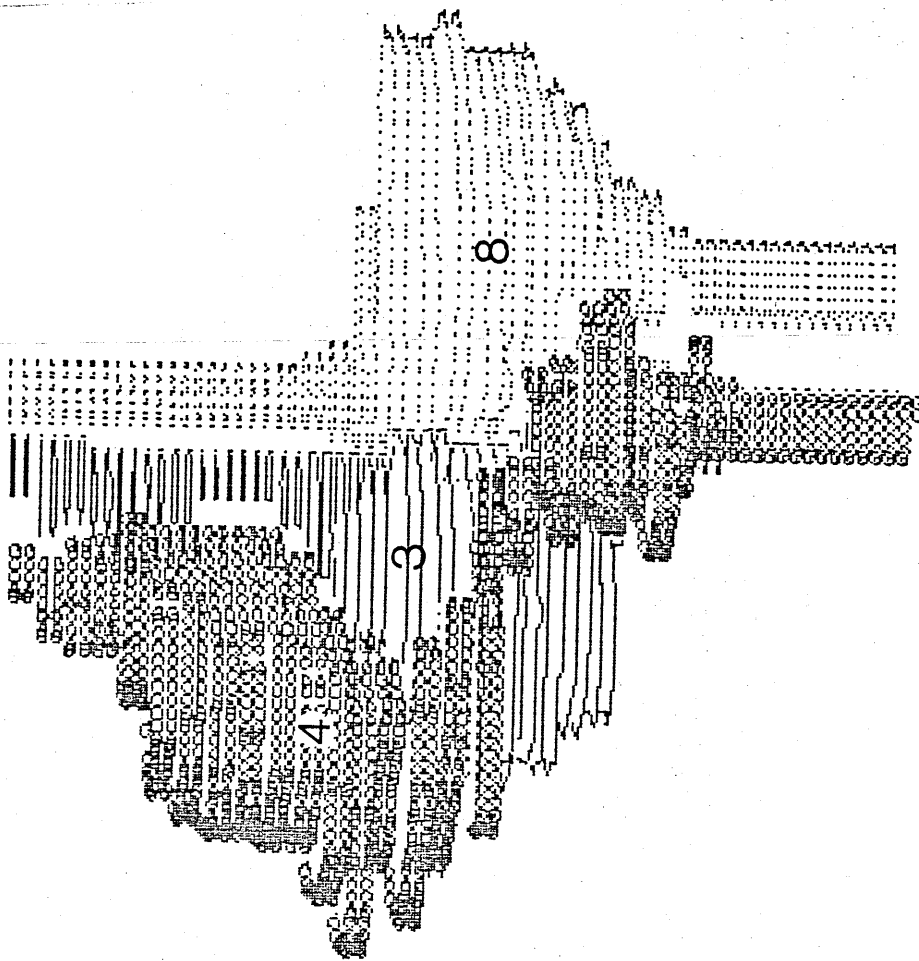


Fig. 3.11b Stage III 0° Posterior is to the left.
Note how cell 4 skirts cell 3 to come to
occupy its correct axonal position.

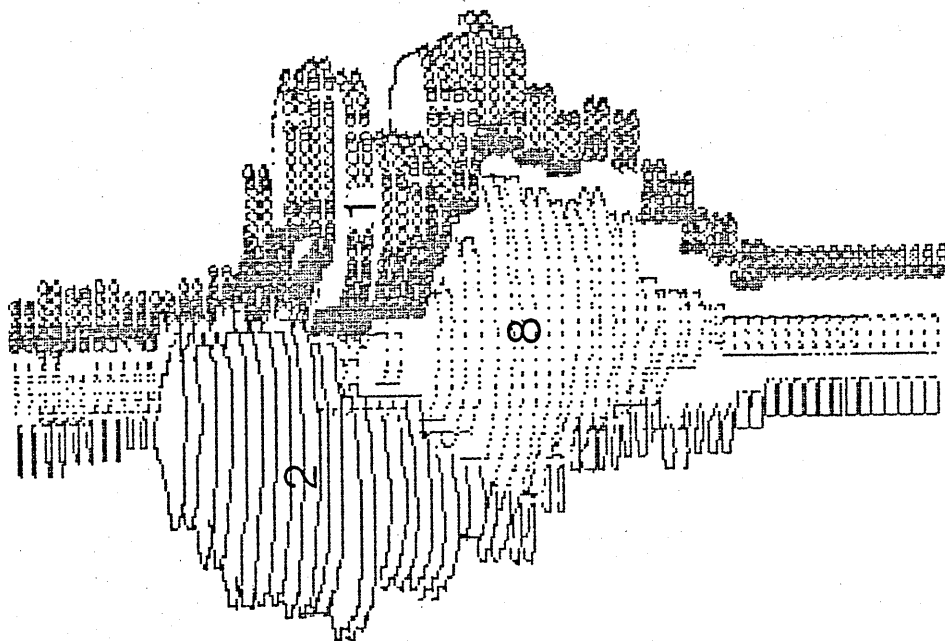


Fig. 3.11c Stage III 90° Polar is to the left. Note how cell-body of 8 juts out anteriorly.

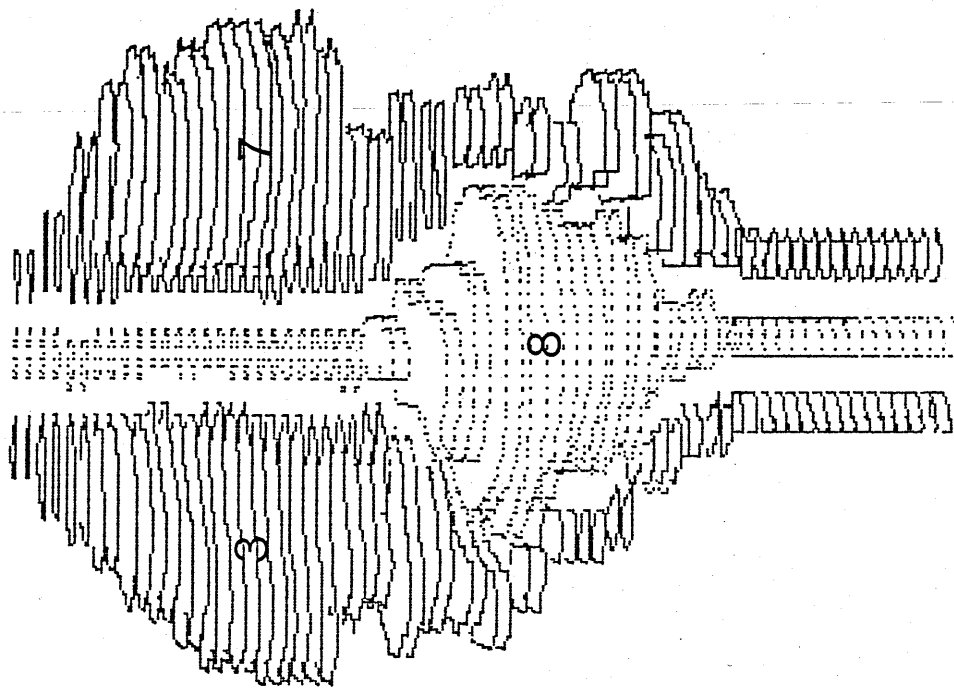


Fig. 3.11d Stage III 90° Polar is to the left. The paired arrangement of cells 3 and 7 about 8.

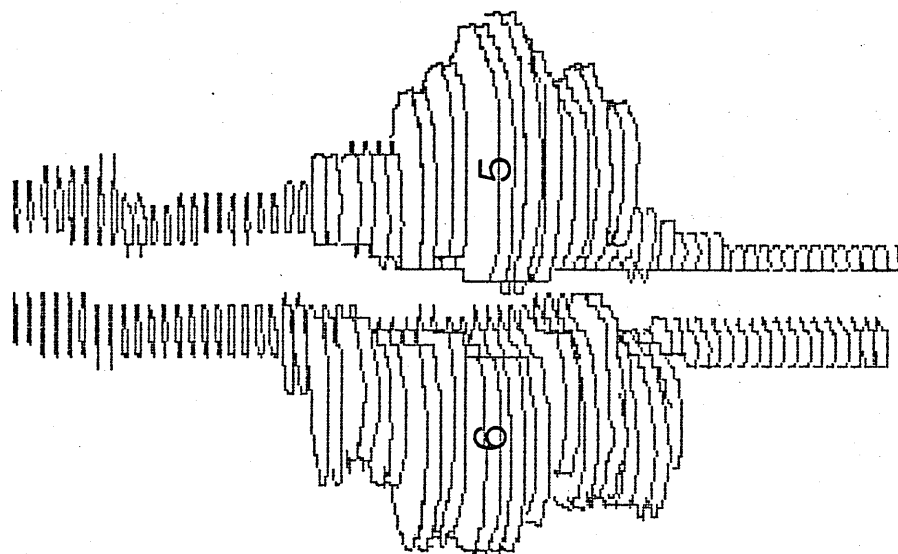


Fig. 3.11e Stage III 270° Equatorial
is to the left. The paired
arrangement of cells 5 and 6

posterior axis and hence show paired behaviour (Fig. 3.11e).

The two non-receptor cell nuclei found in the distal clustering of Stage II have been joined by a further two and the four now lie about the distal projections of the receptor cells above the cell body clustering (Fig. 3.10a). The two non-receptor cell nuclei found distally in Stage II are known to have risen from the pool of non-receptor cell bodies found in the proximal levels of the Stage I ommatidial bundling, but the source of the further two found in Stage III is not as clear owing to the mitotic activity detected in Stage II. Examination of the intermediates between Stages I and II revealed non-receptor cell nuclei distally displaced from the non-receptor cell nuclei pool, and since in Stage I no non-receptor cell nuclei are present at any other location than proximally, then it was concluded that these displaced cells were displaying a distal migration. Although a similar situation was found existing in the intermediates between Stages II and III similar conclusions could not be drawn - the centrally placed non-receptor cell bodies may have risen distally from the proximal regions, or may have resulted from the cell divisions and hence be migrating proximally. Accordingly, the source of the further two non-receptor cell nuclei found distally in Stage III remains unclear, their origin being from either mitotic activity or the proximal non-receptor cell body pool.

3.2 Identification of the mature ommatidial stages and concomitant receptor cells

The information detailed in 3.1.1 has been summarised in Table 3.1 and Fig. 3.12, and the three mature ommatidial stages can be readily

distinguished by the use of this data.

Identification of the individual receptor cells requires knowledge of the anterior direction. In Stages I and II, the cell R_7 is easily distinguishable because it is the only unpaired eccentric receptor cell. Cell R_1 is found to the anterior of cell R_7 and the remaining eccentric receptor cells are arranged consecutively around the central cell with R_6 thus being located to the posterior of cell R_7 (Figs. 3.6; 3.8). Cell identification in Stage III is more complicated owing primarily to the displacement of cell R_4 , and the disruption of the eccentric cellular arrangement by the anterior shift of the central cell body. In distal regions the polar positioning of cell R_4 distinguishes the individual cells of the pair R_3 and R_7 (Fig. 3.10b), and hence the method adopted in Stage I and II cell identification can now be used. But at levels slightly distal to the central cell body, cell R_4 tends to be located on the antero-posterior axis (Fig. 3.10c), and it is not until it begins to skirt about the border of cell R_5 (Fig. 3.10d) that the polar direction indicated. The anterior displacement of the central cell body results in the separation of cells R_1 and R_2 (Fig. 3.10d), cell R_1 is defined as the most anterior equatorially placed cell, and cell R_2 is found opposite on the polar border of the central cell-body and is the most anteriorly placed polar cell. With the exception of cell R_4 , the remaining receptor cells are found consecutively arranged about the border of the central cell with R_7 consequently being immediately posterior to cell R_1 (Fig. 3.10d). At more proximal regions of the central cell body cell R_4 now gains contact with the border of cell R_8 and now occupies the position in the sequence of cells arranged about the central cell as its number indicates.

Having established, at any of the three developmental stages, the cell identities, if the receptor cells are arranged in a clockwise manner about the central cell then the clustering is located in the prospective ventral eye region, conversely on the other side of the line of pattern inversion the prospective dorsal tissue cells are ordered in an anti-clockwise direction.

TABLE 3.1 DISTINGUISHING CHARACTERISTICS OF THE MATURE OMMATIDIAL DEVELOPMENTAL STAGES

	OVERALL LENGTH LUMEN SURFACE TO OPTIC STALK	SHAPE OF CELL BODY CLUSTERING	NUMBER OF DISTALLY PLACED NON-RECEPTOR CELL NUCLEI	NON-RECEPTOR CELL CONTACTS OF CENTRAL CELL	CELL PAIRING	PRESENCE OF 'BOW TIE' ARRANGEMENT OF DISTAL TIPS OF R1,2,6,5,+8	R4 CELL DISPLACED POSTERIORLY FROM CLUSTER DISTALLY & FROM R8 CENTRALLY	POSITION OF R7 CELL BODY IN CLUSTERING	POSITION OF R8 CELL BODY IN CLUSTERING
STAGE I	40-45 μ m	SQUARE	NONE	NONE	R1 & R6 R2 & R5 R3 & R4	NO	NO	PROXIMAL	CENTRAL
STAGE II	35-40 μ m	ELONGATED IN EQUATORIO-POLAR AXIS	2	CENTRALLY NON-RECEPTOR CELLS GAIN CONTACT ANTERIORLY & POSTERIORLY	R1 & R6 R2 & R5 R3 & R4	YES	NO	DISTAL	CENTRAL
STAGE III	30-35 μ m	ELONGATED IN ANTERO-POSTERIOR AXIS	4	CENTRALLY ANTERIOR JUTS OUT OF CLUSTERING & CONTACTS NON-RECEPTOR CELLS	R3 & R7 R6 & R5	YES	YES	DISTAL	ANTERIORLY DISPLACED

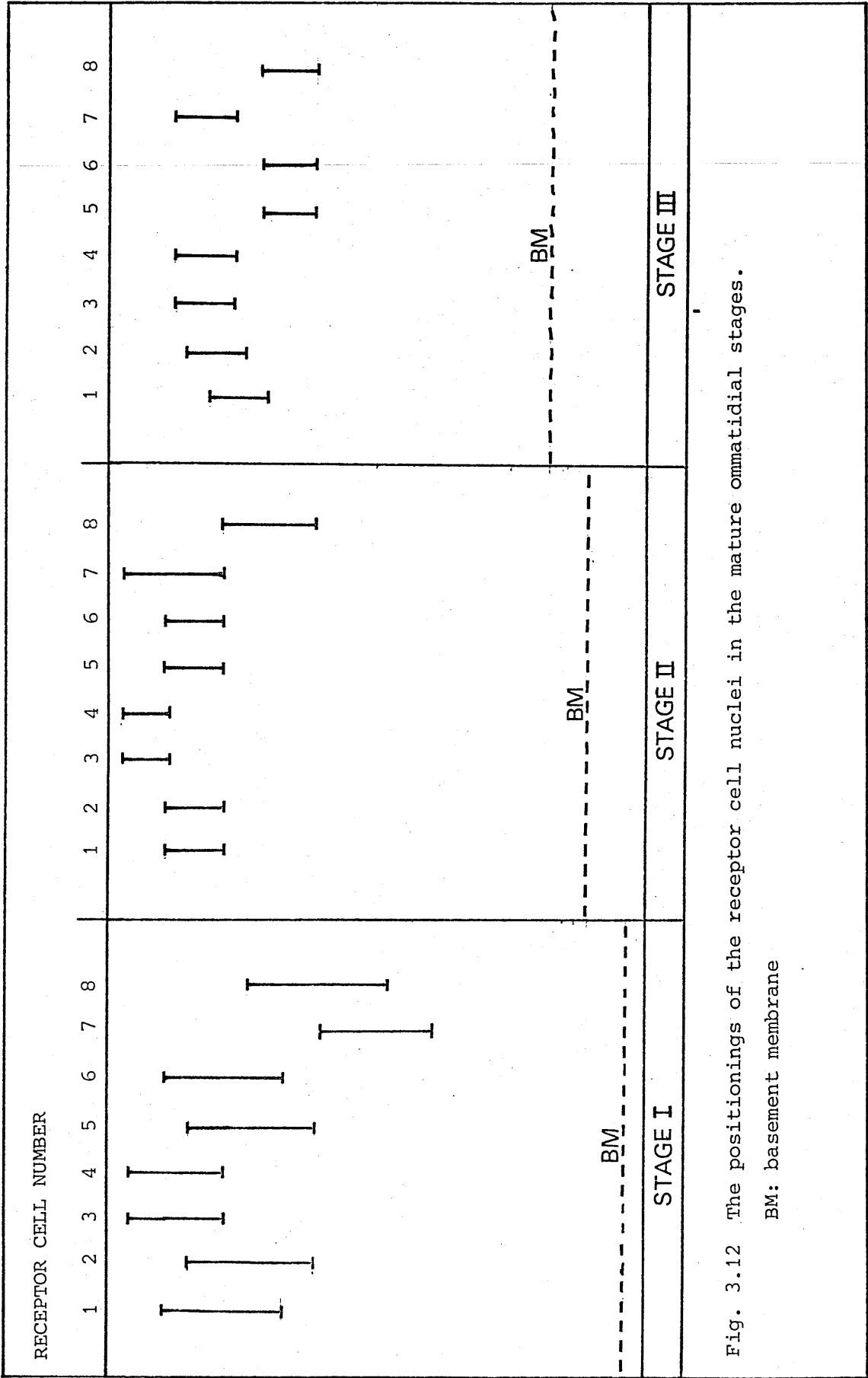


Fig. 3.12 The positionings of the receptor cell nuclei in the mature ommatidial stages.

BM: basement membrane

4. THE RECEPTOR CELL ARRANGEMENT IN THE TISSUE ANTERIOR TO THE MATURE OMMATIDIAL BUNDLES

Having established the constitution of the mature developing ommatidial bundles, the investigation was now directed towards the more anterior tissue, the region of pre-clustering described by Ready et al, (1976) (see Chapter 1, 3.1.2). The pre-cluster of five receptor cells recruits three more such cells to establish the eight cell, mature, ommatidial bundle. Preliminary examination by the serial section technique, in an attempt to identify developing ommatidial bundles with progressively increasing receptor cell complements failed, all pre-clusters examined were found to consist of at least seven, probably eight, clustered cells. Five of the cell-bodies were found distally and were obviously clustered, but in more proximal locations at least two, probably three, cell-bodies were found to be intimately associated with the bundle. The initial indication of this was that seven, or eight cell clusters had, by their distal appearance, been wrongly identified as five cell pre-clusters and the term immature ommatidial bundle will now be used to describe the clusters of this condition.

In the examination of the mature ommatidial bundles a receptor cell was defined as bearing proximally a constituent of a developing axonal bundle containing the full complement of eight projections. But in the immature ommatidial bundles, complete axonal bundles were not necessarily found, and a receptor cell, owing to its immature condition, did not always possess an axonal process and accordingly the developing axon could not be used as the criterion for distinguishing receptor from non-receptor cells. By moving anteriorly from the Stage I ommatidial bundle and scrutinising each immature bundle, it was

found that receptor cell identity could be shown for a particular immature bundle by comparing it with the bundle immediately posterior, establishing the corresponding cells, and then comparing this more mature bundle with its immediate posterior neighbour, and hence in a step wise manner back to the Stage I ommatidial bundle. This technique proved very successful largely due to the fact that the structural changes between adjacent immature bundles was not great and as a result cellular correlation between bundles could be performed fairly easily.

After the examination of the immature tissue from many head discs had been performed it was recognised that the immature stages were far more transitional than the mature i.e. a mature stage could be readily found in most head discs examined, but an immature stage, once characterised, could rarely be found in the same, or another disc. The rapidity of nuclear migrations occurring in the immature tissue meant that the receptor cell bodies, at a particular developmental position, did not persist for sufficient time for it to be accorded stage status. The progressive cellular rearrangement occurring in the immature tissue, however, was quite apparent and consisted of an orderly distal migration by the nuclei of cells $R_{1,6+7}$ from a proximal location accompanied by a distal reorganisation of the remaining five receptor cell-bodies.

The evidence to be presented is taken from a single head disc, firstly, to display the validity of the cell identification technique by the comparative anatomy of adjacent clustering, and secondly, to ensure by tracing anteriorly from one bundle to the next that the correct developmentally regressive series is examined. A considerable

amount of corroborative evidence exists, taken from the investigation of the immature tissue of many head discs, but the evidence presented is considered sufficiently conclusive without reference to supportive information.

Generally, between the MF and the mature ommatidial stages, three to four rows of immature bundles are present. In the series to be described there were three immature bundles with a fourth of specialised nature which will be dealt with separately.

The individual immature bundles have been ordered into the series, A,B,C, the alphabetical series indicating their developmental maturity, and since they will be traced anteriorly towards the MF then they will be dealt with in reverse order.

In the immature clusterings the receptor cells can be distinguished into two groups - $R_{1,6+7}$ and $R_{2,3,4,5+8}$, which will be referred to as equatorial, and polar, cells respectively.

4.1 The Structure of the Three Immature Ommatidial Bundles

Immature Bundle C (IMC)

This clustering was found directly anterior to a Stage I mature ommatidial bundle and in its distal region only the nuclei of the five polar cells are evident, giving the appearance of a five cell pre-cluster. The equatorial border of the cell R_8 is occupied by the distal projections from the cell bodies of cells $R_{1,6+7}$ (Fig. 3.13a).

Examination of sections from more proximal levels reveals that there is a full eight cell bundle, the pre-clustering appearance in the distal region resulting from the nuclei of cells R_1 and R_6 being found more proximally placed than in the Stage I mature bundle. The most distally placed equatorial nucleus is that of cell R_6 , this being in the region of the lower cell body clustering of the polar cells (Figs. 3.13b; 3.14a). More proximally, as the polar cells are beginning to reduce to their axonal processes, the nucleus of cell R_1 is found (Figs. 3.13c; 3.14b), the most proximally placed nucleus being that of cell R_7 (Figs. 3.13d; 3.14c). This cell body persists to levels where all other receptor cells are only represented by their axonal projections (Fig. 3.13e), a very similar situation to that found in the Stage I mature bundle (Fig. 3.6e). All eight of the bundle receptor cells bear developing axons which project into the developing optic stalk. Since all of the receptor cells bear an unequivocal axonal projection then this bundle could be regarded as mature, but it has been decided to regard it as immature on the grounds that the axon projecting from cell R_7 becomes displaced from the other seven axonal projections for a distance (Fig. 3.13f) before occupying its normal position in the axonal grouping (Fig. 3.17g) i.e. at certain levels within the axonal projections, a complete axonal bundle cannot be observed.

All polar cell nuclei are found more distally placed than they are in the Stage I mature bundle (Fig. 3.19), those of cells R_3 and R_4 (Fig. 3.14d) being more distal to those of cells R_2 and R_5 (Fig. 3.14e). The cell pairing evident in the mature stages is not yet established, the posterior member of a prospective cell pair is, to a lesser extent with the polar cells and a greater extent with the equatorial

FIGURE 3.13

Sections taken from Immature Bundle C. All receptor cells are numbered.

Fig. 3.13a x 10,000. 5 μ m below distal surface. The five distally placed polar nuclei give the appearance of a pre-cluster. Note the distal projection of R₁, 6 + 7 about the equatorial border of R₈.

Fig. 3.13b x 10,000. 10 μ m below distal surface. R₆ is the first equatorial nucleus to be encountered.

Fig. 3.13c x 10,000. 16 μ m below distal surface. The nucleus of R₁ is found as the polar cells are beginning to reduce to their axonal projections.

Fig. 3.13d x 10,000. 22 μ m below distal surface. The most proximally positioned nucleus is R₇.

Fig. 3.13e x 10,000. 28 μ m below distal surface. The nucleus of R₇ persists to levels where other cells are represented by their axonal projections.

Fig. 3.13f x 10,000. 33 μ m below distal surface. The projection from R₇ becomes displaced from its normal positioning.

Fig. 3.13g x 10,000. 40 μ m below distal surface. A correctly ordered axonal bundle now projects to the optic stalk.

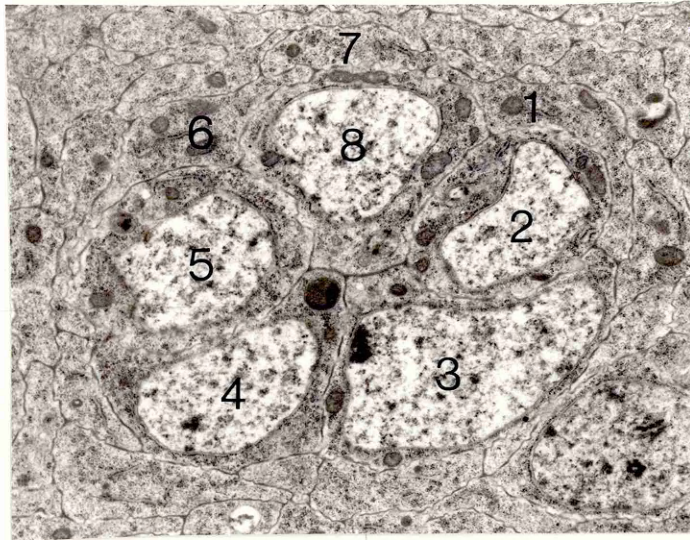


Fig. 3.13a

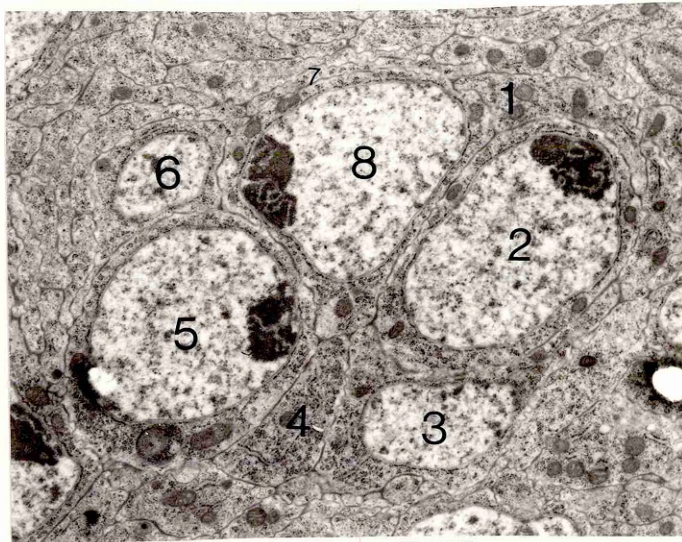


Fig. 3.13b

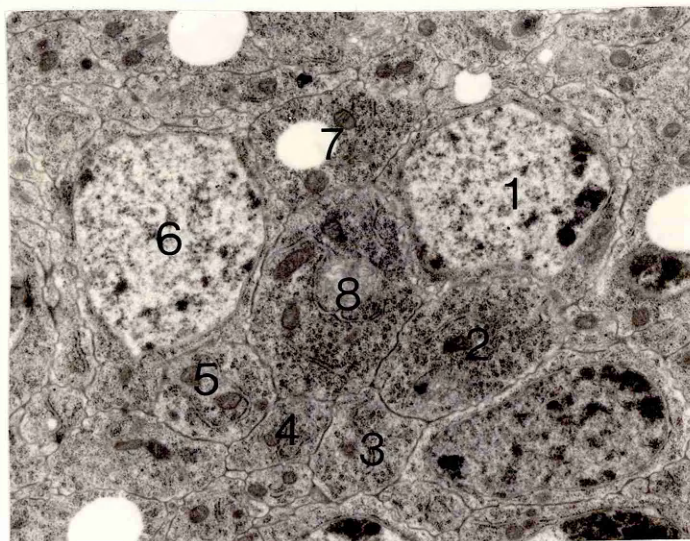


Fig. 3.13c

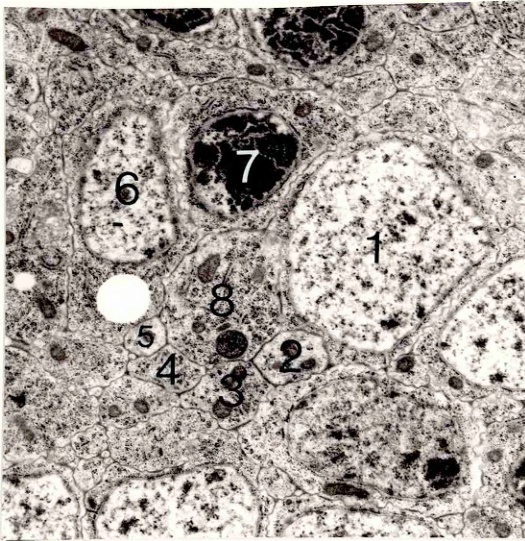


Fig. 3.13d

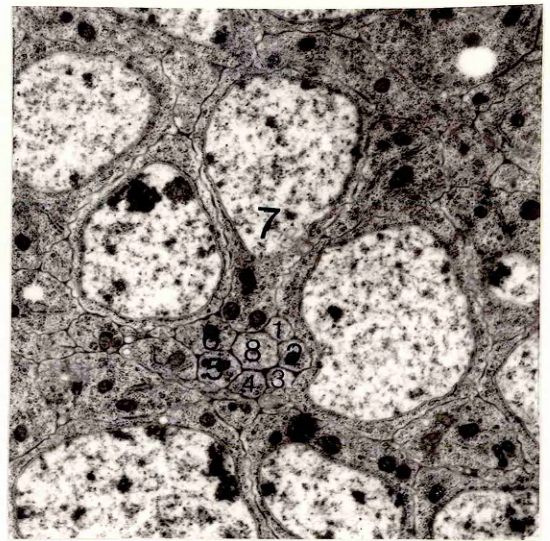


Fig. 3.13e

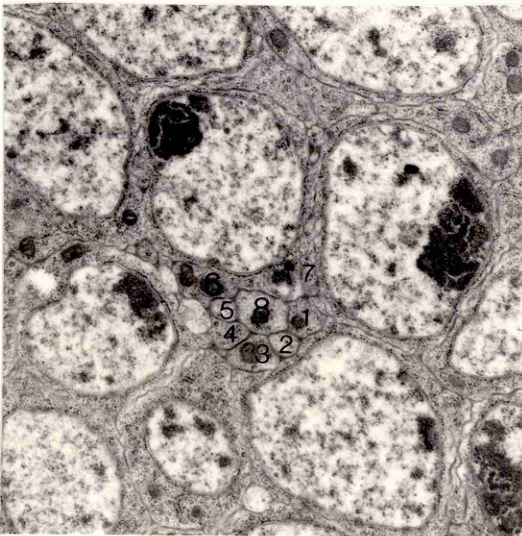


Fig. 3.13f

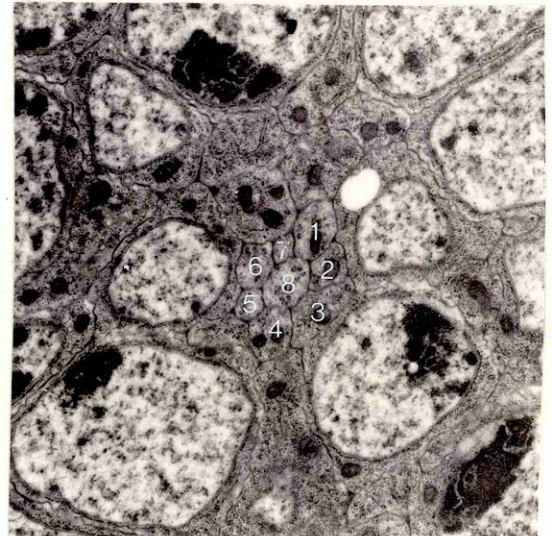


Fig. 3.13g

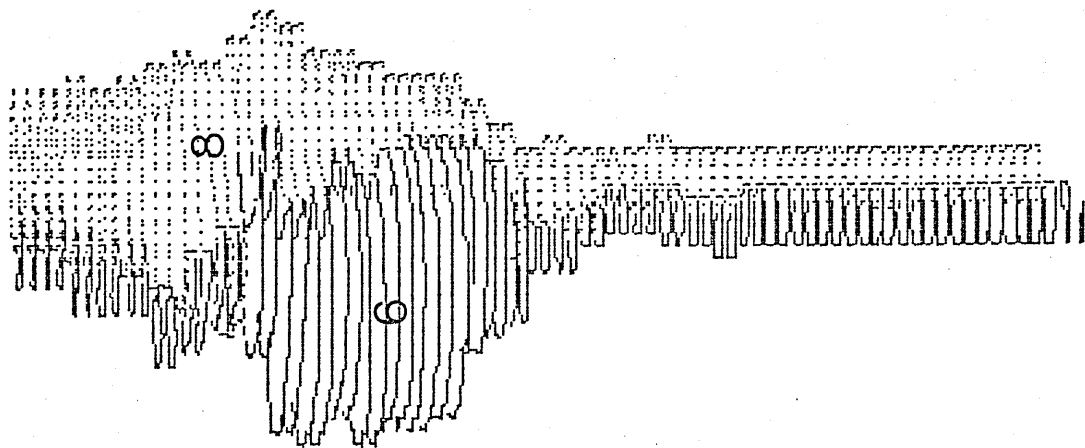


Fig. 3.14a IMC. 270°. Equatorial is to the left. The positioning of cell 6

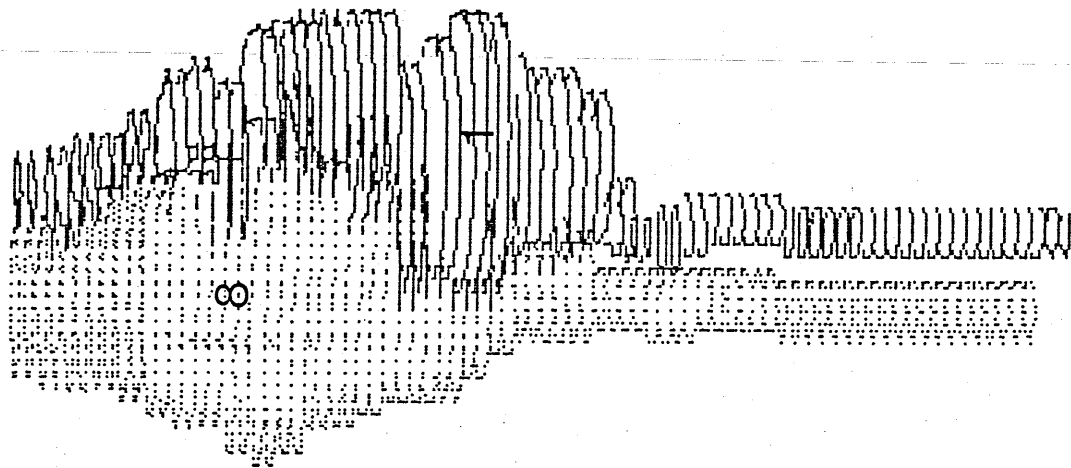


Fig. 3.14b IMC. 67°. Polar is to the left. The positioning of cell 1 about 8.

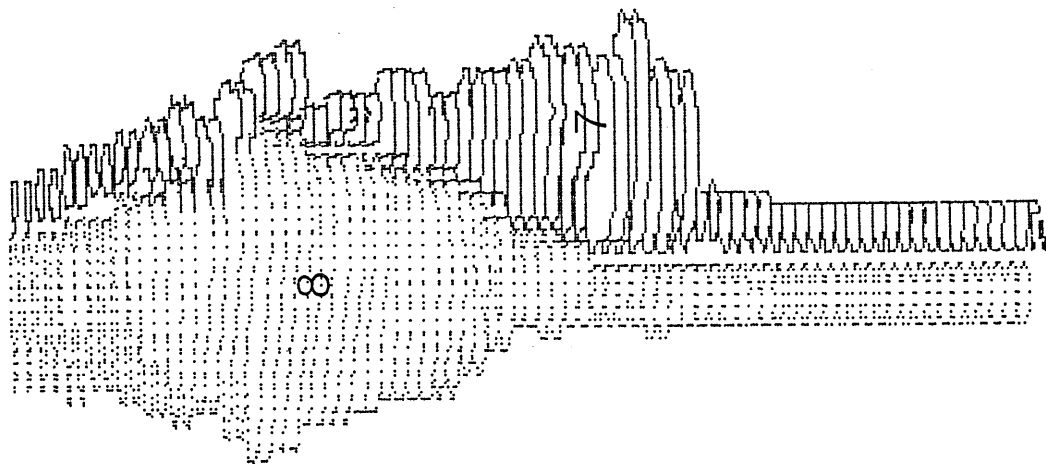


Fig. 3.14c IMC. 90°. Polar is to the left. The positioning of cell 7 about 8.

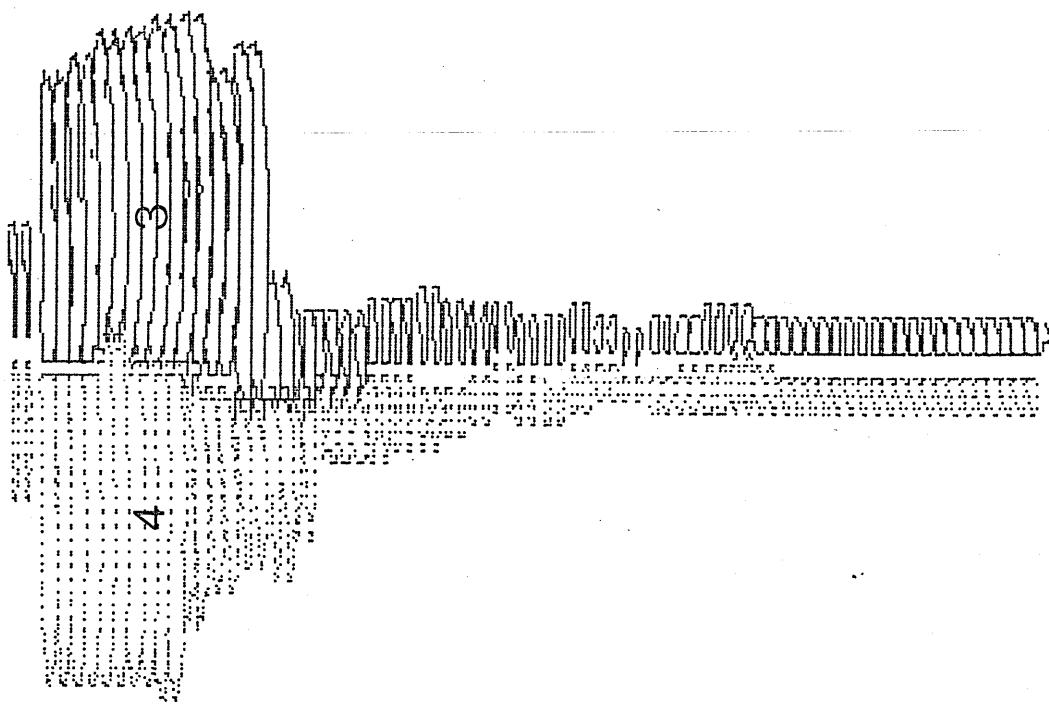


Fig. 3.14d IMC. 0°. Posterior is to the left. The arrangement of cells 4 and 3.

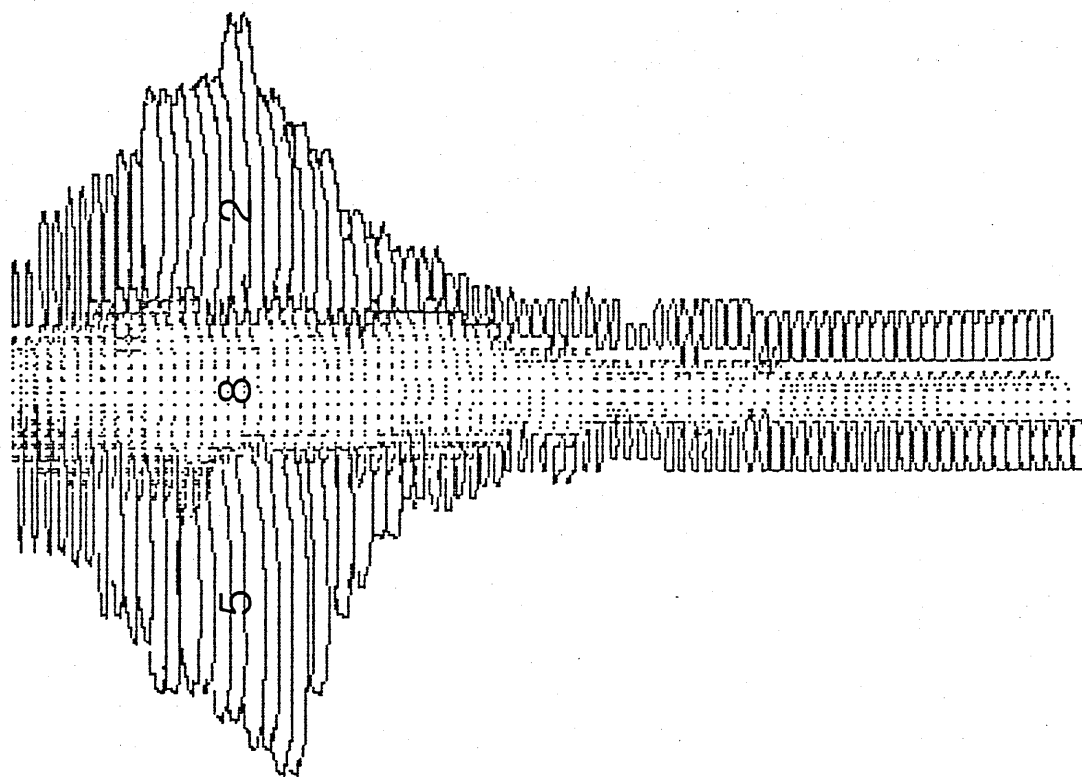


Fig. 3.14e IMC. 0°. Posterior is to the left.
The arrangement of cells 5 and 2
about 8.

cells, always found to be the more distally positioned of the two.

As in the mature stages, all retinal cells appear to be joined to their neighbours, at a level slightly below the lumen surface, by the adherens type junctions - a situation found in all the immature ommatidial bundles.

Examination of Figs. 3.13 and 3.14 shows clearly that cell identification can be performed in this immature bundle with ease, the essential difference between this immature bundle and its later mature counterparts is the more proximal location of the equatorial nuclei, this providing the key in the examination of the more immature clusterings.

Immature Bundle B (IMB)

Moving anteriorly from the Immature Bundle C (IMC) the Immature Bundle B (IMB) was found and here the equatorial cell bodies are more proximally placed and the distally located polar cell bodies, arranged somewhat differently, extend to a more proximal level (Fig. 3.19).

In the distal region of the clustering, the projections to the lumen surface from the equatorial cell bodies can be clearly seen about the equatorial border of the cell R_8 (Fig. 3.15a). The nucleus of cell R_4 , as in IMC, is the most distally placed but that of cell R_3 is found proximal to its location in IMC, the cell body of R_5 being found more distal in the more immature bundle (Figs. 3.16d,e; 3.19).

Moving proximally from the distal region of the clustering, the nucleus of cell R_6 is the first of the equatorial nuclei to become evident (Fig. 3.15b), being slightly more proximally placed than in IMC (Fig. 3.16a), and as in IMC the cell body of cell R_1 is the intermediately placed member of the equatorial cell bodies. With the exception of being more proximally placed, the arrangement of the nuclei of cells R_1 and R_6 are found in a similar location to IMC, but a major difference exists with the cell body of R_7 , it being now precluded from contact with the central cell body. The distal projection from the cell body of R_7 occupies its expected location on the central cell border (Fig. 3.15a,b,c) but with the increased diameter, as the cell body is encountered, it becomes occluded from contact with the central cell body by cells R_1 and R_6 (Fig. 3.15d). Comparison between IMB and IMC (Figs. 3.15e; 3.13e) reveals that in the more immature bundle, where the cell body of R_7 is more proximally located, the central axon is surrounded by the axons from cells R_{1-6} , the central projection not being available for contact by the cell body of R_7 . There is a short proximal projection from the cell body of R_7 which nestles between the axonal projections from cells R_1 and R_6 , but still contact with the central axon does not occur (Fig. 3.15f). Some 6-8 μ m below its cell body the projection from cell R_7 terminates and an axonal bundle with only seven constituents projects through the most proximal regions to the developing optic stalk (Fig. 3.15g).

In IMC all eight receptor cells could be identified without difficulty but in IMB because cell R_7 loses contact with the central cell then one of the criteria used for receptor cell identification is not being fully satisfied. However, there can be little doubt

FIGURE 3.15

Sections taken from Immature Bundle B. All receptor cells are numbered.

Fig. 3.15a x 10,000. 6 μ m below distal surface. The distal projections from R₁, 6 + 7 occupy the equatorial border of R₈.

Fig. 3.15b x 10,000. 12 μ m below distal surface. R₆ is the most distally placed equatorial nucleus.

Fig. 3.15c x 10,000. 20 μ m below distal surface. The nucleus of R₁ is encountered as the polar cells are beginning to reduce to their axonal projections.

Fig. 3.15d x 10,000. 25 μ m below distal surface. R₇ is the most proximally positioned nucleus, note its exclusion from contact with R₈.

Fig. 3.15e x 10,000. 33 μ m below distal surface. The nucleus of R₇ persists to levels where other cells are represented by their axonal projections. R₇ is still excluded from contact with R₈.

Fig. 3.15f x 10,000. 38 μ m below distal surface. A short proximal projection emanates from R₇ (arrow).

Fig. 3.15g x 10,000. 40 μ m below distal surface. An axonal bundle containing only seven elements projects into the optic stalk.

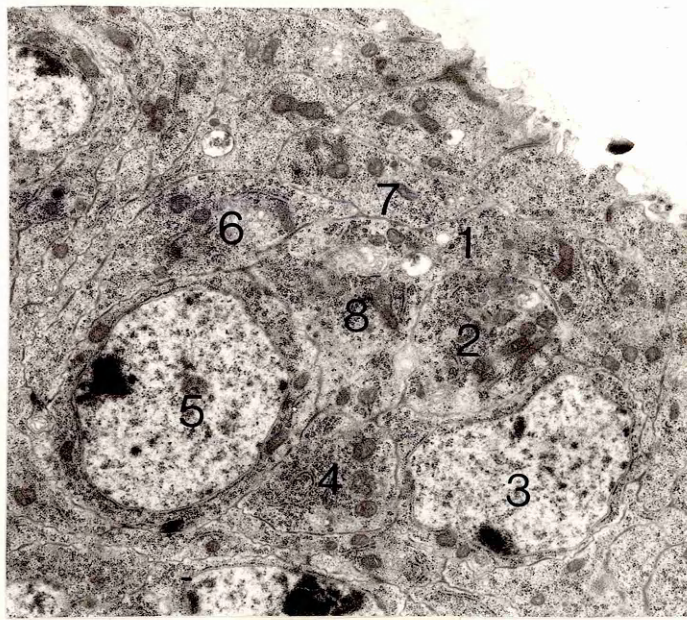


Fig. 3.15a

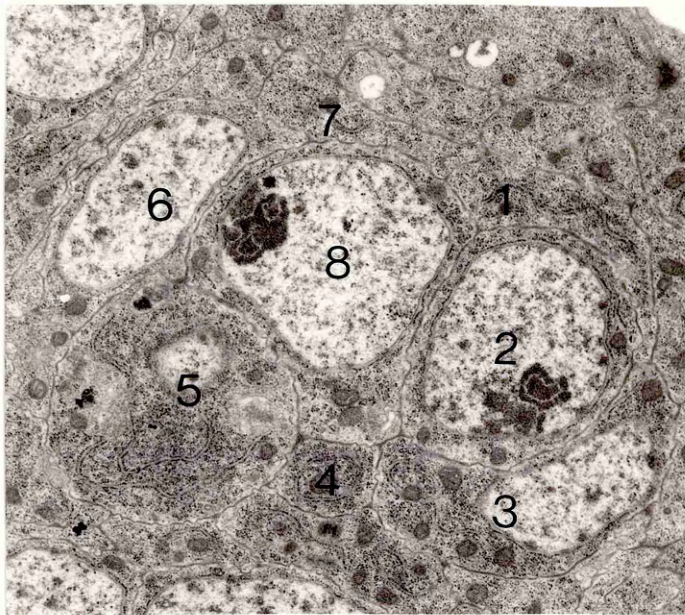


Fig. 3.15b

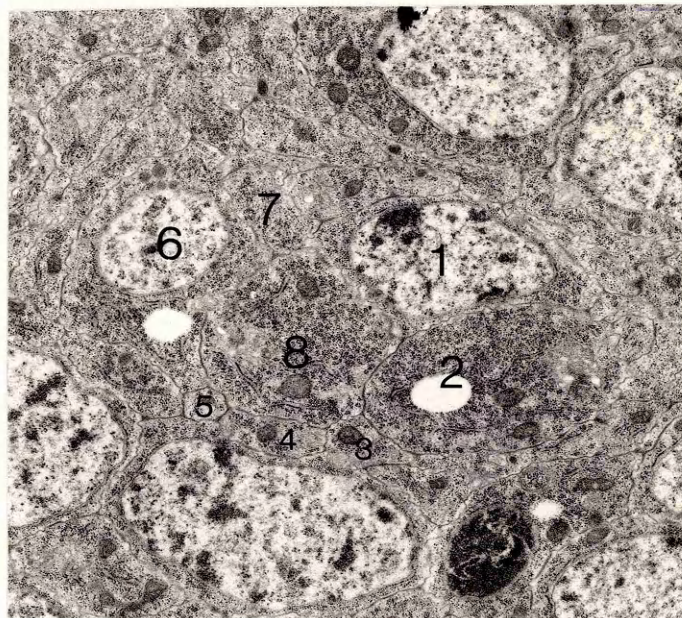


Fig. 3.15c

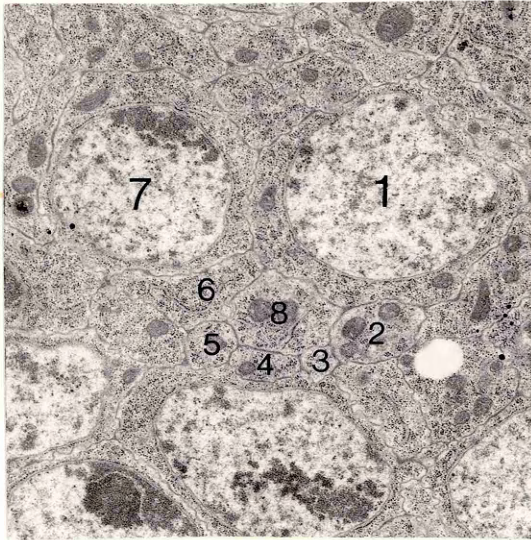


Fig. 3.15d

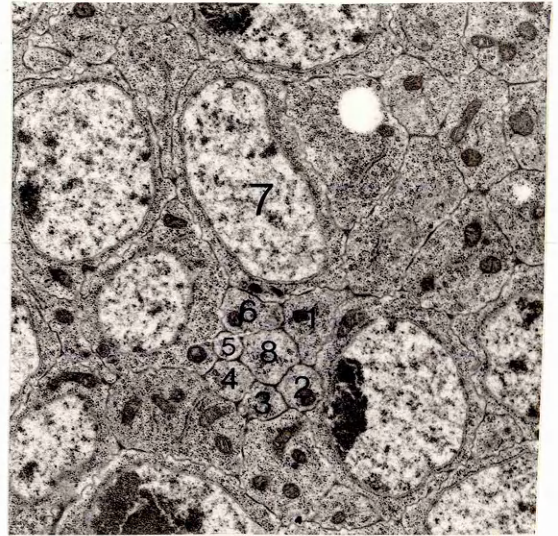


Fig. 3.15e

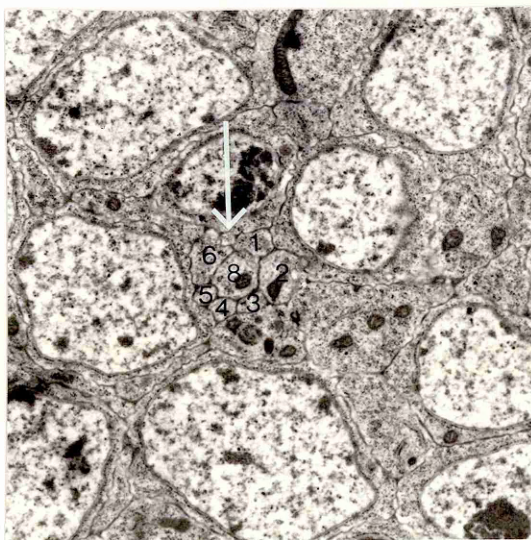


Fig. 3.15f

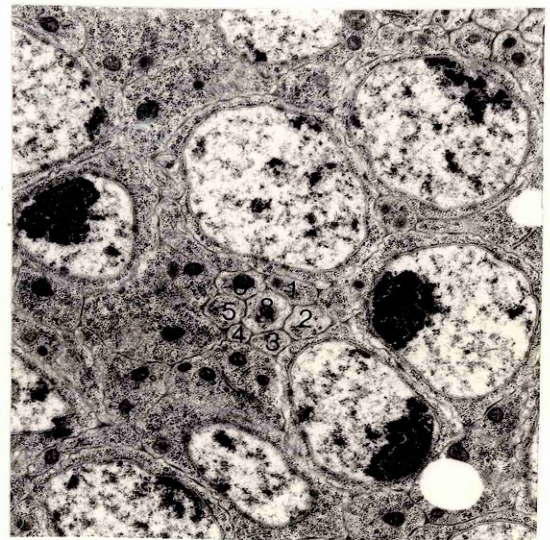


Fig. 3.15g

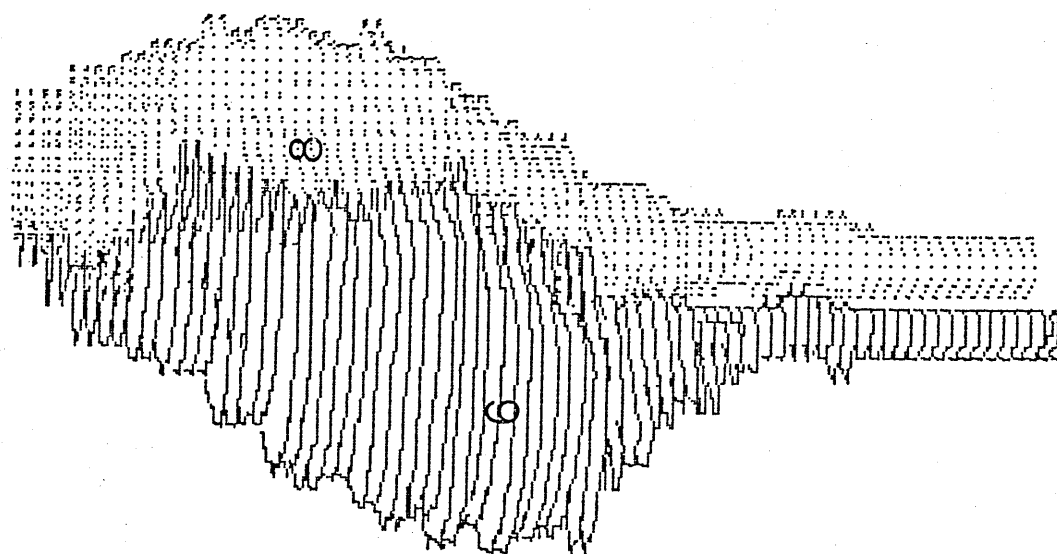


Fig. 3.16a IMB. 270°. Equatorial is to the left. The positioning of cell 6 about 8.

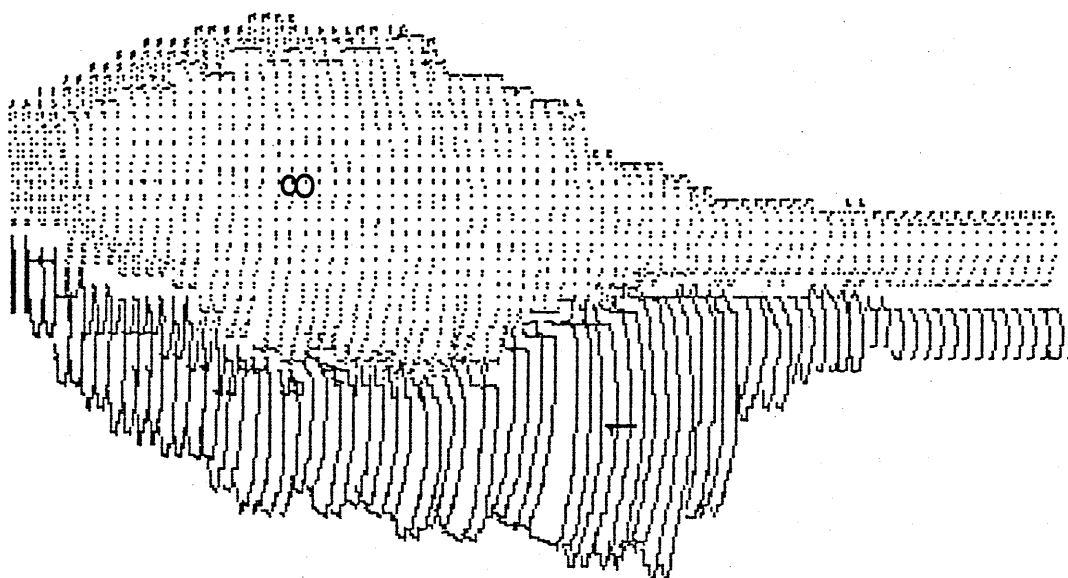


Fig. 3.16b IMB. 225°. Equatorial/anterior is to the left. The positioning of cell 1 about 8.

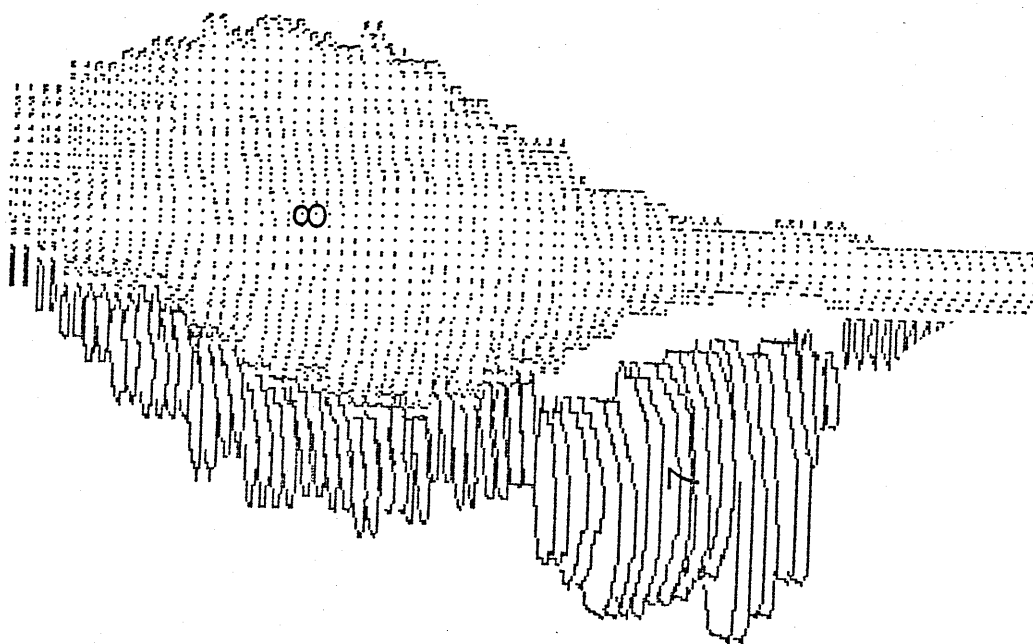


Fig. 3.16c IMB. 270°. Equatorial is to the left. The positioning of cell 7 about 8.

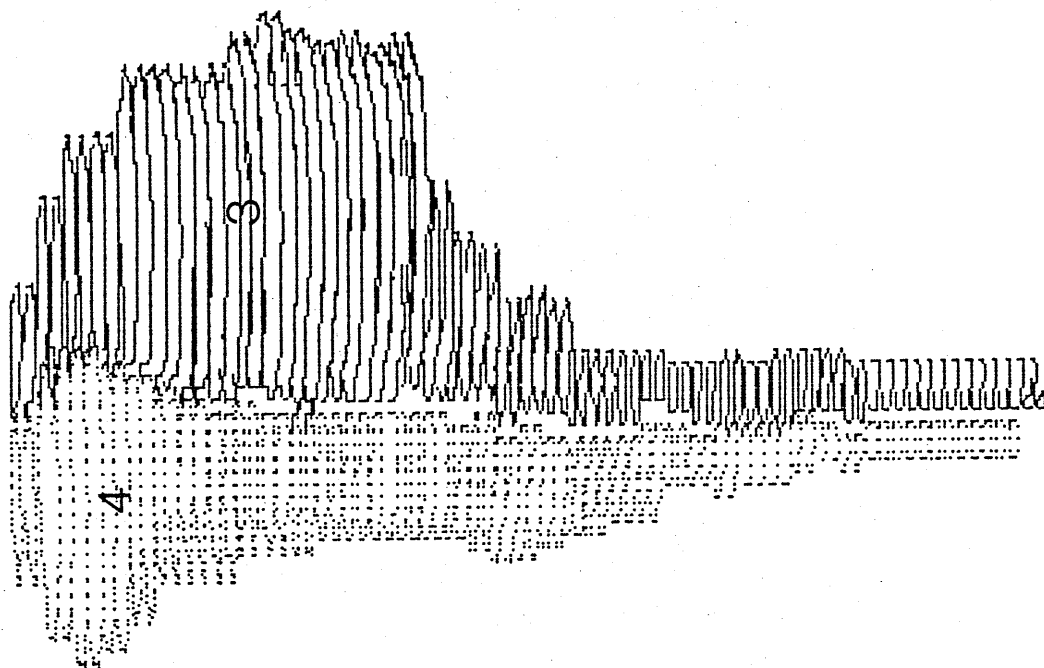


Fig. 3.16d IMB. 0°. Posterior is to the left. The arrangement of cells 4 and 3.

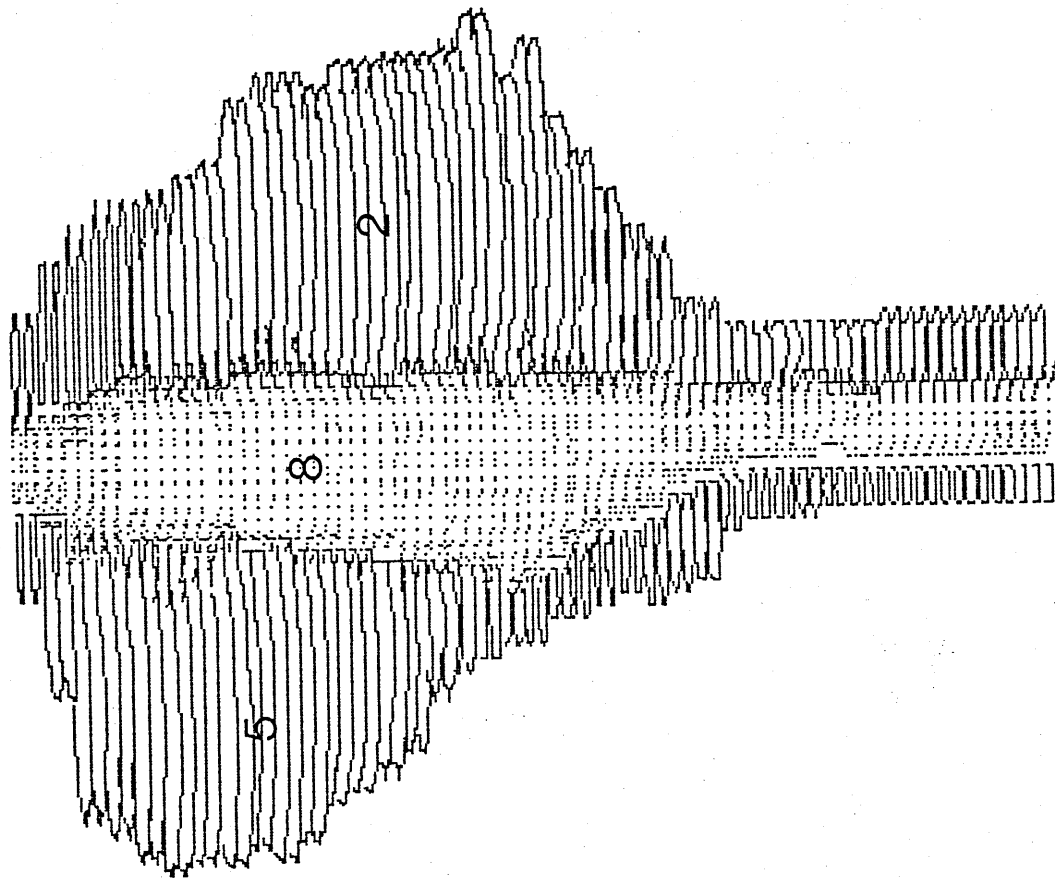


Fig. 3.16e IMB. 0°. Posterior is to the left.
The arrangement of cells 5 and 2
about 8.

that the cell in question is R_7 . As will be explained in greater detail later, no cell division is occurring in this region of the tissue, accordingly cell R_7 must be present, its cell body, from indication from IMC, is expected proximally - as it is, its distal projection occupies the correct clustering position, its cell body is in the correct location being only occluded from contact with the central cell, and proximally a projection from the cell body is beginning to grow into the developing axonal bundle. Hence, all eight receptor cells of IMB have been identified, the two essential features of this bundle are that the equatorial cell bodies are found more proximally located than in IMC, and that cell R_7 , peculiar among its counterparts, has not yet developed an axonal process into the optic stalk.

Immature Bundle A (IMA)

The Immature Bundle A (IMA) was found anterior and adjacent to IMB and is the most immature of this developmental series. Receptor cell identification is slightly more difficult than in the more mature bundles but sufficient evidence exists to allow cell identification with the same degree of confidence.

In the later immature ommatidial bundling, with the exception of the axonal bundle of IMC and the region of cell body of R_7 and axonal bundle of IMB, the central cell is always surrounded by the eccentric receptor cells and a non-receptor cell never gains contact with it.

But in the distal region of IMA the equatorial cells do not completely occupy the equatorial border of the central cell, hence, non-receptor cells gain contact, and accordingly the cells occupying the equatorial border of the central cell cannot, automatically, be afforded equatorial receptor cell status. However, without examining the proximal sections, the equatorial cells can be identified with a reasonable degree of certainty. Fig. 3.17a is a section from the distal region of the clustering and four cells that border the central cell can be identified as not being polar receptor cells. Cell R_1 can be readily identified by its occupation of the anterior wing of the equatorial border of the central cell and also by its contact with cell R_3 (Fig. 3.17a). It is a characteristic of the cell R_1 to contact cell R_3 in the immature bundles, particularly at the more distal levels, resulting in a complete envelopment of cell R_2 by receptor cells (Figs. 3.13a; 3.15a,b). The immature ommatidial bundles are asymmetrical in that cell R_6 does not contact cell R_4 as cells R_1 and R_3 do, and hence this phenomenon cannot be used for cell R_6 identification, but the cell bordering cell R_5 and occupying the expected position on the posterior wing of the equatorial border of the central cell (see Figs. 3.13; 3.15) can be confidently assumed to be R_6 . Of the two remaining cells, one occupies a considerably larger portion of the central cell equatorial border (Fig. 3.17a) and this cell can be assumed to be R_7 - on this information alone the identification of cell R_7 is the one performed with least confidence of the three. Examination of the more proximal sections provides the evidence which confirms the assumptions made from the information available from the distal region. As cell R_7 is traced proximally it is found to displace the cell found between it and cell R_6 distally, and comes to border cell R_6 itself (Fig. 3.17b).

FIGURE 3.17

Sections taken from Immature Bundle A. All receptor cells are numbered.

Fig. 3.17a x 10,000. 10 μ m below distal surface. Four non-polar cells can be seen bordering R₈ equatorially, one (cross) is a non-receptor cell.

Fig. 3.17b x 10,000. 14 μ m below distal surface. The non-receptor cell (cross) becomes displaced from contact with R₈ by R₇.

Fig. 3.17c x 10,000. 18 μ m below distal surface. R₇ loses contact with R₈, being found between R₁ and R₆.

Fig. 3.17d x 10,000. 20 μ m below distal surface. R₆ nucleus is the most distally placed of the equatorial cells.

Fig. 3.17e x 10,000. 32 μ m below distal surface. The proximal positioning of the nuclei of R₁ and R₇ results in section of the bundle containing no nuclei.

Fig. 3.17f x 10,000. 35 μ m below distal surface. The nuclei of R₁ and R₇ are found highly proximally positioned. Note that cell R₆ now also loses contact with R₈ at this depth.

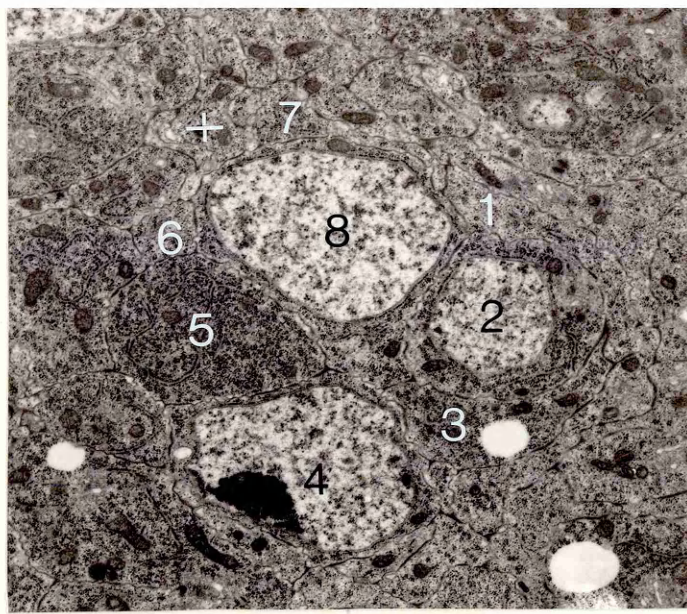


Fig. 3.17a

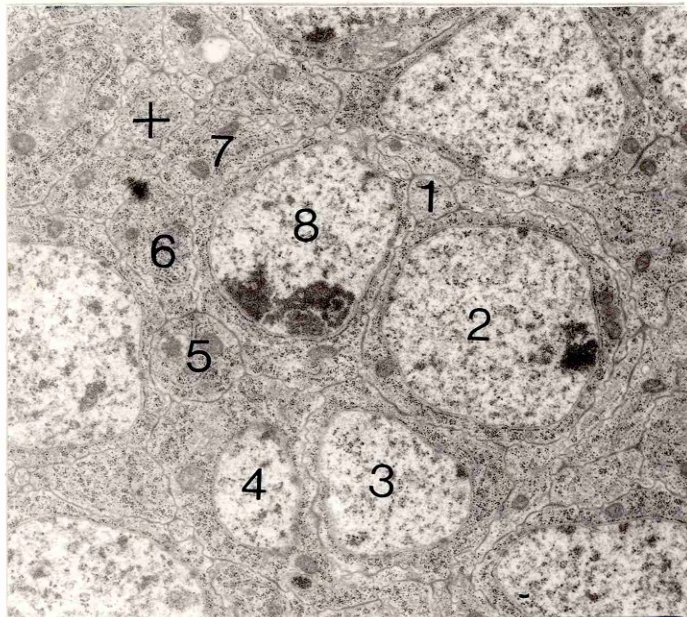


Fig. 3.17b

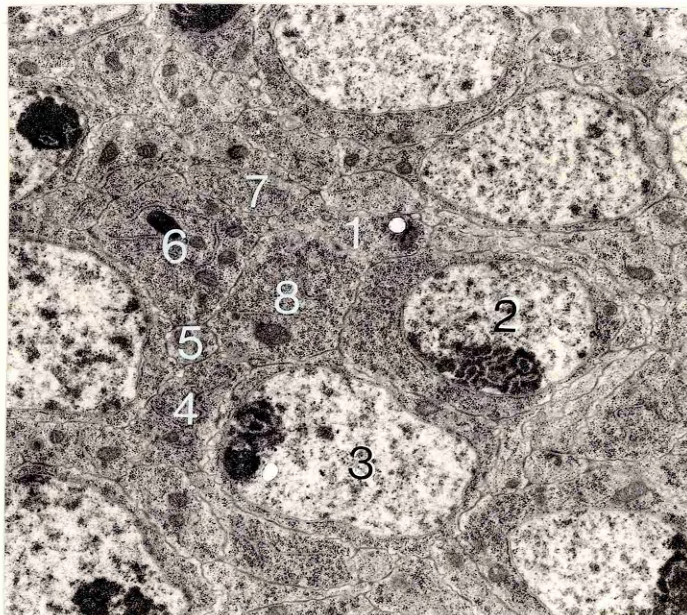


Fig. 3.17c

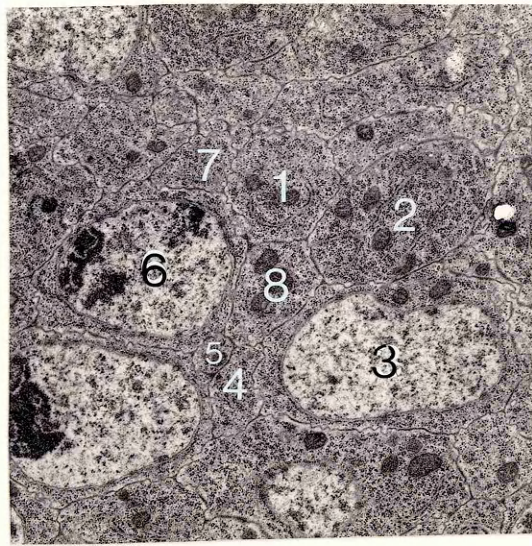


Fig. 3.17d

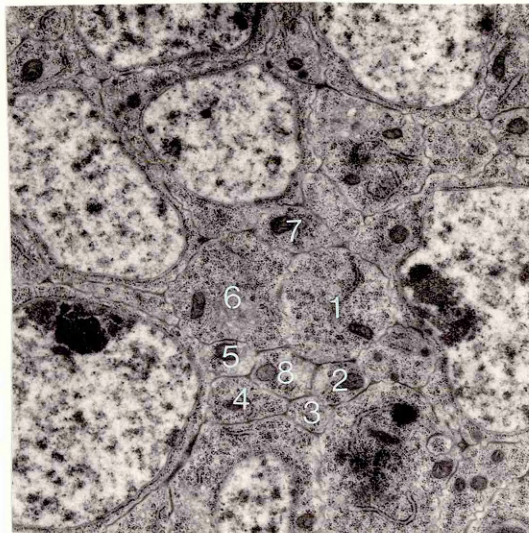


Fig. 3.17e

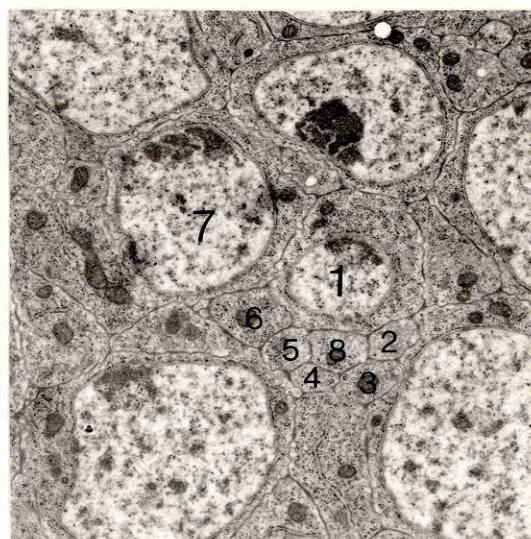


Fig. 3.17f

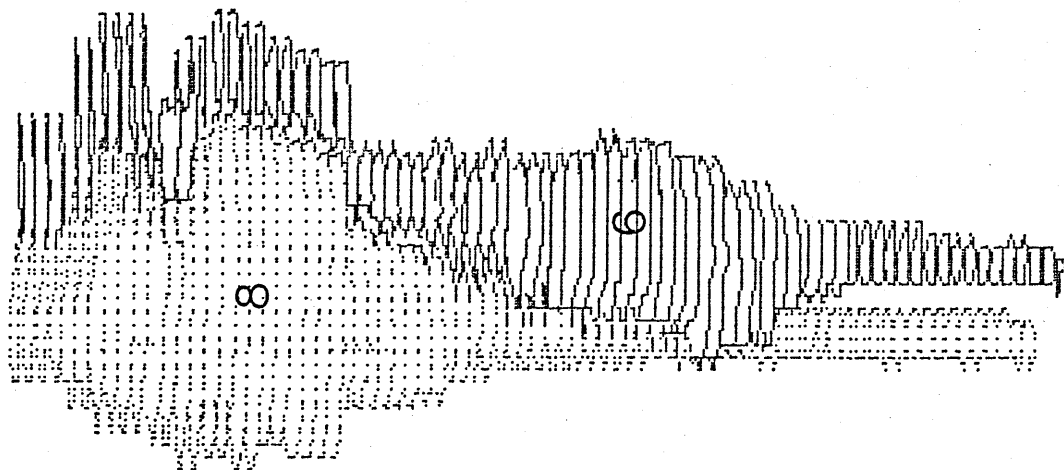


Fig. 3.18a IMA. 180°. Anterior is to the left.
The positioning of cell 6 about 8.

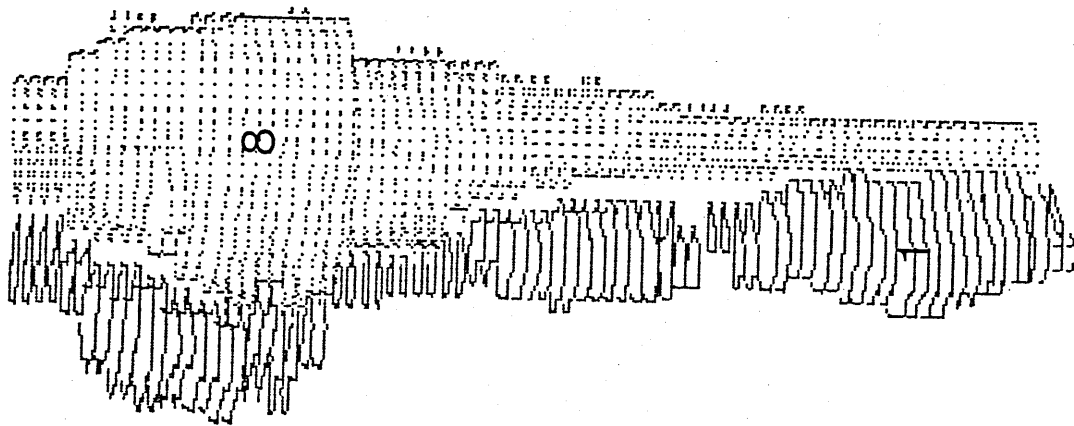


Fig. 3.18b. IMA. 213°. Anterior is to
the left. The positioning
of cell 1 about 8.

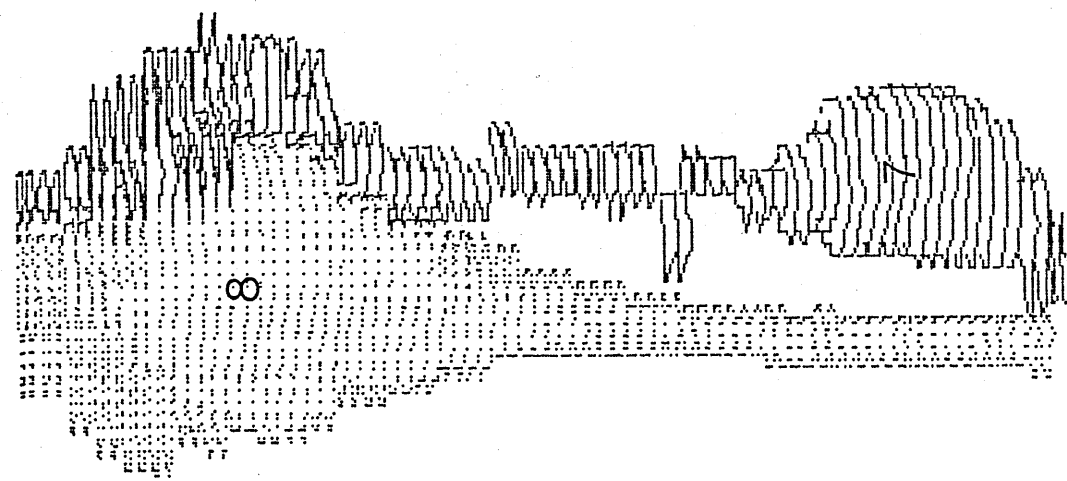


Fig. 3.18c IMA. 90°. Polar is to the left. The positioning of cell 7 about 8.

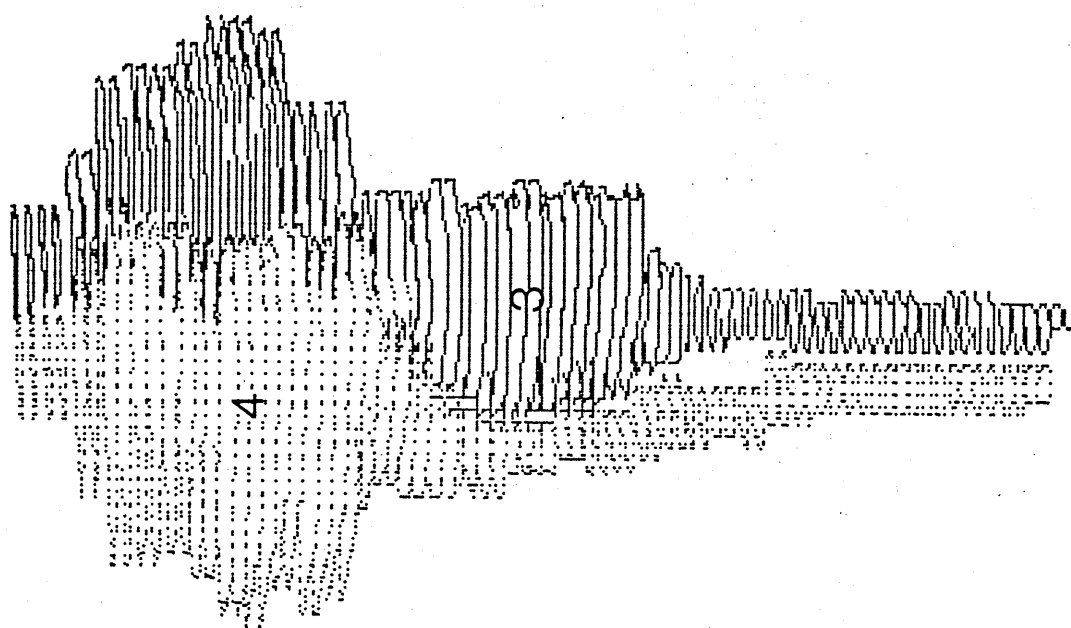


Fig. 3.18d IMA. 337°. Posterior is to the left. The arrangement of cells 4 and 3.

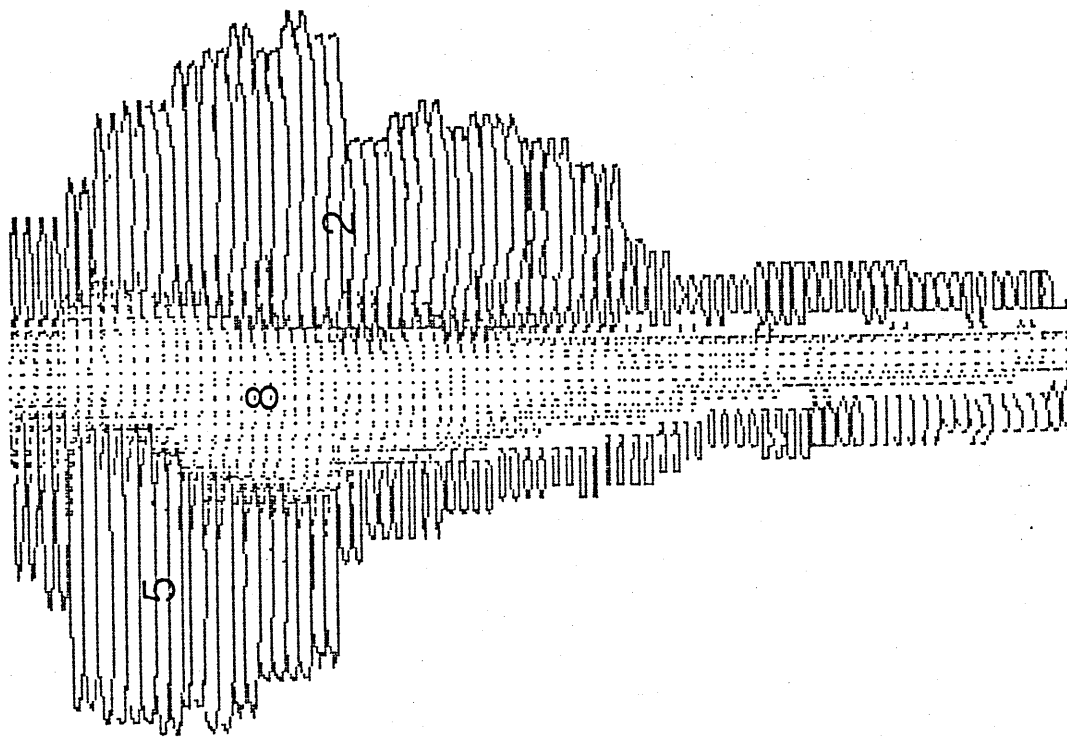


Fig. 3.18e IMA. 0°. Posterior is to the left. The arrangement of cells 5 and 2 about 8.

Cell R_1 does not lose contact with the central cell throughout the entire length of the bundle, a similar situation existing with the cell R_6 , this cell only becoming displaced from the central cell at a very proximal level where the cell-bodies of R_1 and R_7 are present (Fig. 3.17f). Cell R_7 loses contact with the central cell at a proximo-distal level similar to that found in IMB and is located in its expected position between cells R_1 and R_6 (Fig. 3.17c,d,e,f).

The essential differences between IMA and IMB are that the polar cell grouping extends to a more proximal depth (Figs. 3.18d,e; 3.19), and the equatorial nuclei are found considerably more proximally located in IMA. Again the most distally placed of the equatorial cell-bodies is that of cell R_6 (Figs. 3.17d; 3.18a) but the nuclei of cell R_1 , previously found distal to that of cell R_7 is now found at the same, very proximal, level (Figs. 3.17f; 3.18b,c).

Owing to technical difficulties it was not possible to accurately trace the cells of this cluster below the very proximal level described, but with the knowledge of the situation in similar bundles it is suspected that all the polar cells, and possibly cell R_6 , project into the developing optic stalk.

4.2 The Development of the Immature Bundles

The immature bundles have been described in their developmentally regressive order to display the validity of the cell identification technique employed. Having established cellular identity in each of

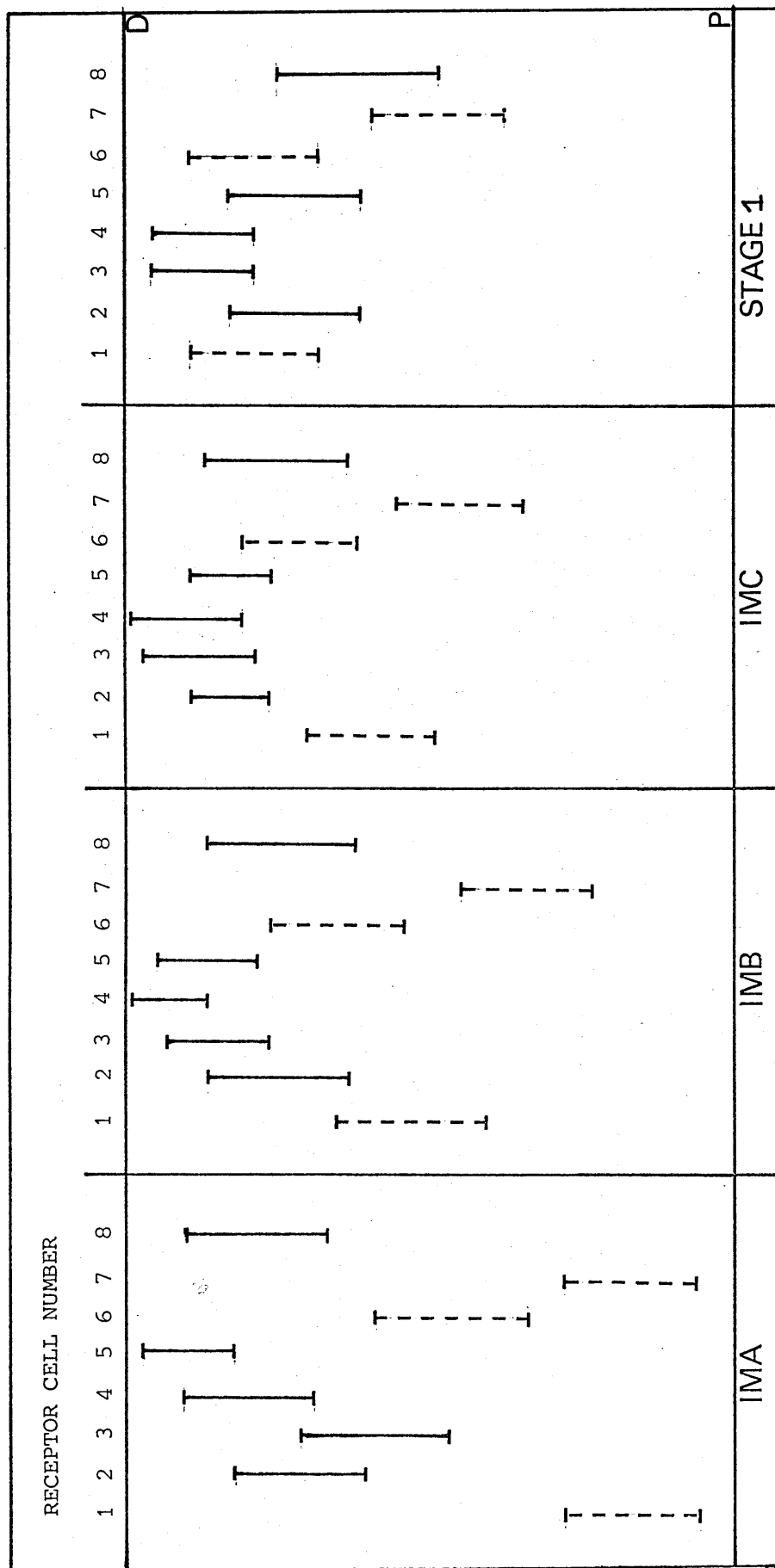


Fig. 3.19 Diagram showing the changes in receptor cell nuclei positioning during immature development.
Equatorial nuclei are dotted.

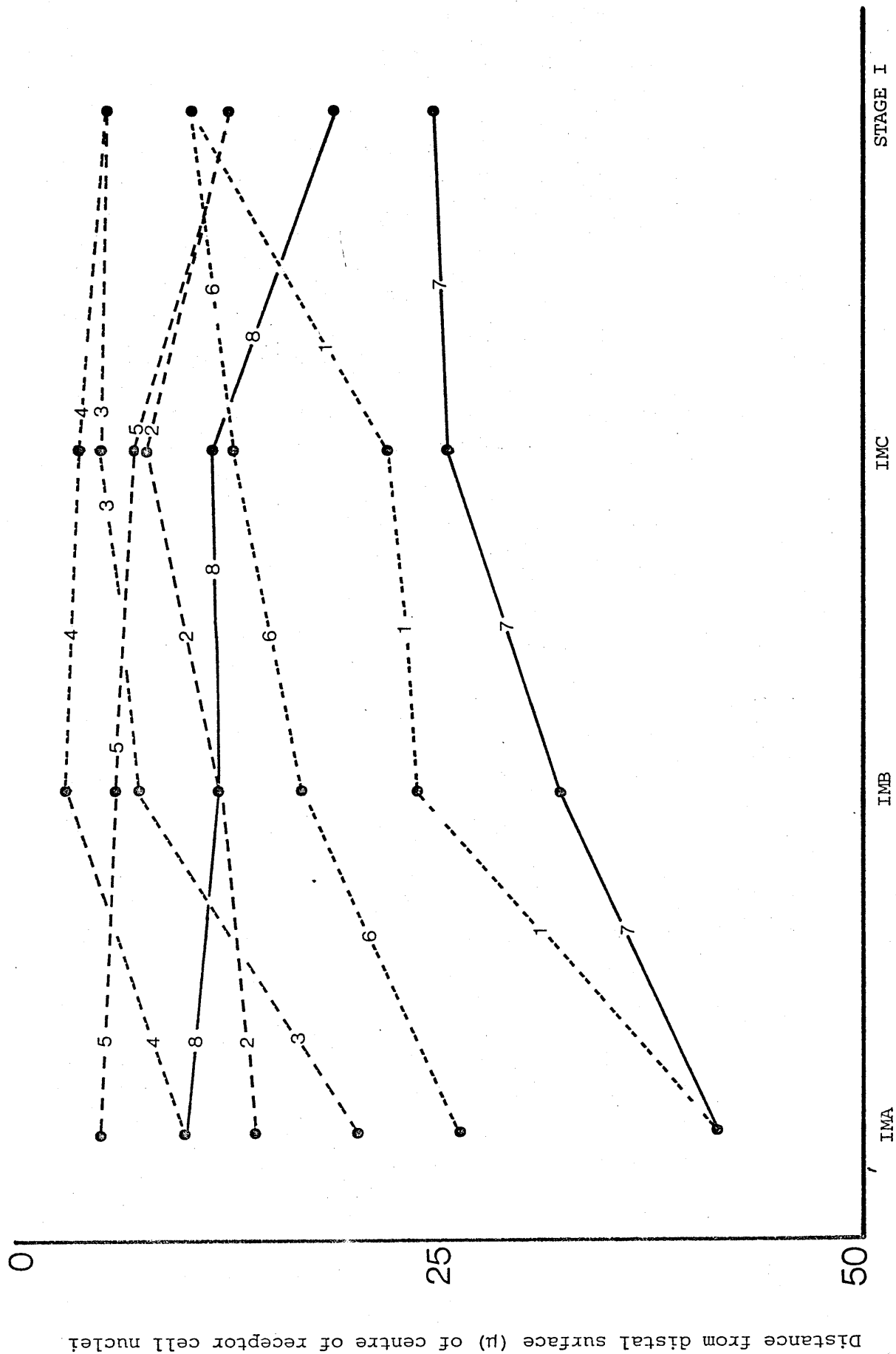


Fig. 3.20 Nuclear migrations occurring during immature development

the bundles, their development in the correct temporal sequence can now be discussed.

The cell bodies of the equatorial cells are initially found proximally, that of cell R_6 being somewhat more distal than the other two, and throughout their immature development they move distally to come to occupy their locations in the mature Stage I ommatidial bundle (Figs. 3.19; 3.20). Conversely, the polar cell bodies are initially found, at various levels, in the distal regions of the tissue, and, although there is obvious movement by each of the cell bodies in the proximo-distal axis, all persist in the distal region and rearrange to form the polar element of the Stage I ommatidial bundles as the equatorial nuclei rise from the proximal levels (Figs. 3.19; 3.20).

Of all six receptor cells which come to pair in the Stage I ommatidial bundle, that which is the posteriorly placed of the two has, during the immature development, the more distally placed cell-body of the pair, i.e. R_6 , R_5 and R_4 are always found distal to their respective pairs R_1 , R_2 and R_3 (Figs. 3.19; 3.20).

4.3 The Structure of the Retinal Tissue in the Region of the Second Mitotic Wave

The MF can be easily located in sections taken in the disto-proximal plane, it is an obvious furrow in the retinal tissue, but when the retinal field is sectioned in the medio-lateral plane, although the centre of the MF is quickly identifiable as an indentation at the edges of the section, its anterior and posterior extent cannot be easily

established. The physical furrowing of the tissue is accompanied by the proximal location of all retinal cell nuclei, the presence of distally placed nuclei, both anteriorly and posteriorly, delimits the extent of the MF. When using the serial section technique, the positioning of the retinal cell nuclei in and around the MF provide the key for establishing whether, or not, the tissue under scrutiny is located within the MF.

The equatorial receptor cells are known to be generated by the second mitotic wave (see Chapter 1, 3.1.4) which has been described as occurring posterior to the MF. The immature ommatidial bundles, previously described, were found to occupy the region between the mature ommatidial bundles and the MF, but throughout the immature ommatidial bundle series no mitotic activity, which can be readily detected under the electron microscope, was ever observed. This finding not only proves that the equatorial cells must be present in the immature ommatidial bundles, but also questions where the second mitotic wave occurs.

When the tissue directly anterior to IMA was examined it was found that when moving posteriorly through the sections a number needed to be examined before nuclei were encountered, hence, it was concluded that this tissue was in the posterior region of the MF. It was in this location that the mitotic activity was detected. The mitosis was found confined to a very small region, approximately two ommatidial rows, and accordingly the equatorial cells, and hence the eight cell ommatidial bundles, are being generated in a very confined area of tissue. Because the eight cell bundles are produced so rapidly then the comparative anatomy technique between the mitotic tissue and the immature ommatidial

bundles could not be performed with the confidence found earlier and hence, it was recognised that the limitation of this technique had been reached. However, information could still be derived from the analysis of the sections and many important findings have accrued.

In the most distal regions of the tissue no nuclei are present, but the clustering of the polar cells is still, if not as obviously as before, detectable. The most distally placed nucleus is that of cell R_5 and it can be clearly seen to be a member of a cluster of at least five, the polar, cells (Fig. 3.21a). Occupying the posterior wing of the equatorial border of cell R_8 , and contacting cell R_5 , is a cell appearing very similar by location to the cell R_6 in the immature ommatidial bundles, but since mitosis is occurring in this region it cannot be concluded that this is in fact cell R_6 (Fig. 3.21a,b).

In the distal part of the clustering, anterior and equatorial to the cell R_8 , a small distal projection of a cell is evident (Fig. 3.21a), which, slightly more proximally, expands to a relatively large diameter (Fig. 3.21b), this being a cell in the later stages of the mitotic cycle and shows peculiar characteristics. Slightly more proximal to the section shown in Fig. 3.21b cytokinesis can be seen to be in operation, the constriction of the cleavage furrow can be seen, which under higher magnification clearly carries microtubules (these filaments being in the order of 25 nm) which are probably the remnants of the spindle apparatus (Fig. 3.21c,d). The constriction separates the cell into two components and below this level each moiety appears as an individual cell defined by its own plasma membrane (Fig. 3.21e).

Proximal to this, each moiety carries a nucleus with a fully formed double membrane in which the chromatin is condensed - these nuclei being clearly distinguishable from those of the polar cells which reached their terminal division in the first mitotic wave (Fig. 3.21f). Some 4-5 μ m below the level of their nuclei the 'feet' of this cell terminate, the overall appearance of this dividing cell is displayed in Fig. 3.22. This cell, which is in the later stages of the division cycle, is displaying the operation of a unilateral cleavage furrow, the perhaps indicating that the underlying contractile ring is contracting asymmetrically, the cleavage furrow first appears proximally and then moves distally until the division is complete (Fig. 3.23).

As previously explained, in the distal regions of this tissue the polar cells can be seen clustering, but as the cells are traced proximally, although the polar cells persist in their contacts with each other, the clustering is not easily distinguishable (Fig. 3.21g) and more proximal to this situation the clustering is invaded by other cells, the arrangement of four of the polar cells about the fifth (R_8) no longer existing and the essential clustering appearance is lost (Fig. 3.21i).

Most of the cells found directly equatorial to the distal polar cell clustering in the more proximal regions gain contact with the equatorial border of the central cell, at least eight have been recognised to do this and hence attempts at identification of the equatorial receptor cells cannot be performed. At certain levels some of the cells appear to occupy the expected location of a particular

equatorial receptor cell, but at other levels contact with the polar cells, which initially indicated the cell identity, is lost and accordingly any attempt to identify a particular equatorial cell could only be highly speculative.

No cell in this region of tissue projects into the optic stalk, and as a result even the identity of the polar cells must be questioned, but their appearance in the distal clustering is so similar to that of IMA that their identity is assumed with appropriate reservation.

Anterior to the tissue described above a cell of large diameter occupying the entire distal half of the tissue was present, its nucleus still bearing the typical double membrane but its chromatin condensed and hence, owing to its large size, was assumed to be pre-mitotic (Fig. 3.21i). Other cell nuclei were found very proximally placed, indicating the centre of the MF, and any cell clustering could not be detected. In the anterior region of the MF neither mitotic activity nor clear cell clustering was evident.

The adherens type junctions found in the retinal tissue posterior to the MF are still present in the MF itself, even the cell in the later stages of the mitotic cycle is connected to its neighbours by this type of junction (Fig. 3.21b) and examination of tissue more anterior reveals that throughout the MF, below the lumen surface, many adherens type junctions are evident.

FIGURE 3.21

Section from a bundling in the second mitotic wave. All polar cells are numbered, R_6 is also numbered but the identity of this cell is not held with the confidence held for the polar cells.

- Fig. 3.21a x 10,000. 12 μ m below distal surface. Note the typical arrangement of the polar cells. The cross indicates the distal projection of the mitotic cell.
- Fig. 3.21b x 12,500. 15 μ m below distal surface. The mitotic cell (M) is relatively large at this level.
- Fig. 3.21c x 12,500. 18 μ m below distal surface. Note the constriction of the cleavage furrow which separates the cell into two components (M).
- Fig. 3.21d x 22,000. High power of the constriction separating the two parts of the cell (M). Note the microtubules (arrow) which are probably the remnants of the spindle.
- Fig. 3.21e x 10,000. 20 μ m below distal surface. The two proximal moieties of the mitotic cell (M) appear as individual cells.
- Fig. 3.21f x 10,000. 25 μ m below distal surface. Each moiety of the mitotic cell (M) can be seen to carry nuclei with condensed chromatin.
- Fig. 3.21g x 10,000. 30 μ m below distal surface. The clustering appearance begins to be lost at this depth.
- Fig. 3.21h x 10,000. 45 μ m below distal surface. The polar cell arrangement of four cells surrounding a fifth no longer persists and the essential clustering appearance is lost. The 'feet' of the mitotic cell terminate distal to this section.
- Fig. 3.21i x 10,000. The large pre-mitotic cell found anterior to the clustering.

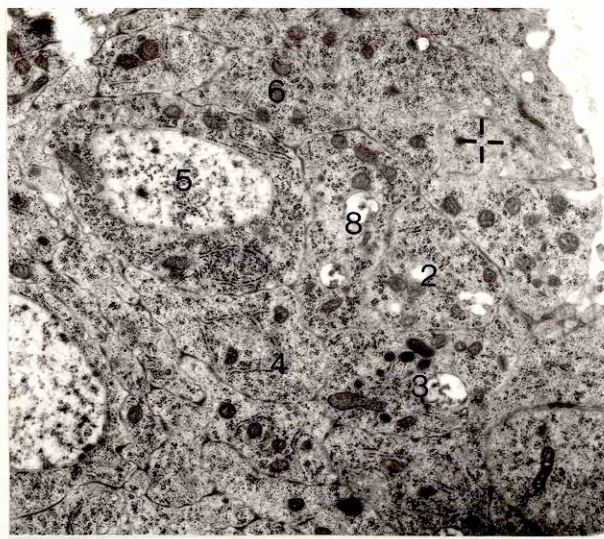


Fig. 3.21a

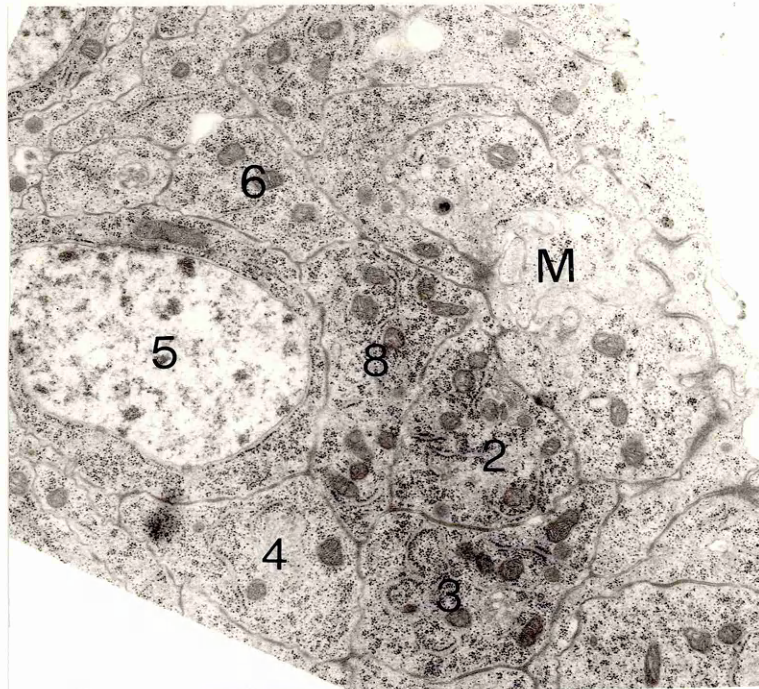


Fig. 3.21b

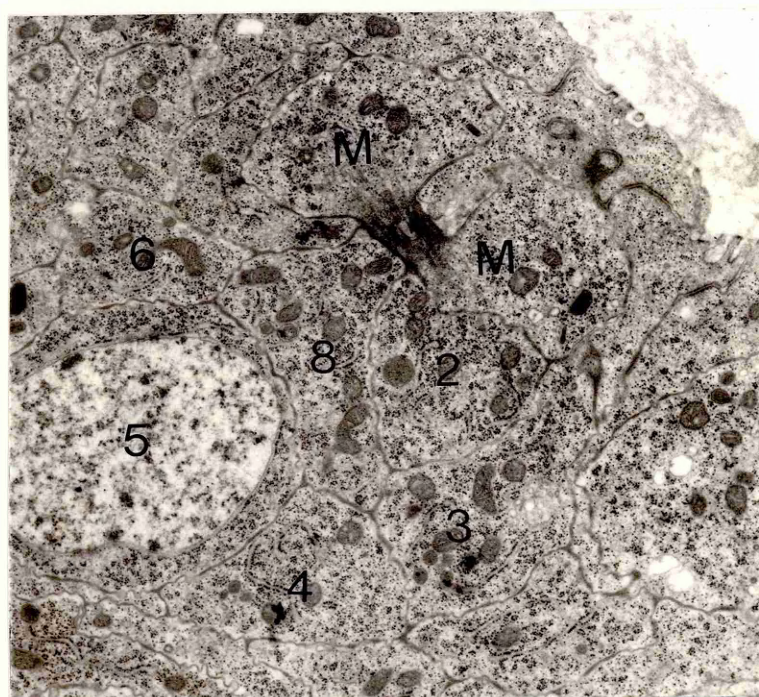


Fig. 3.21c

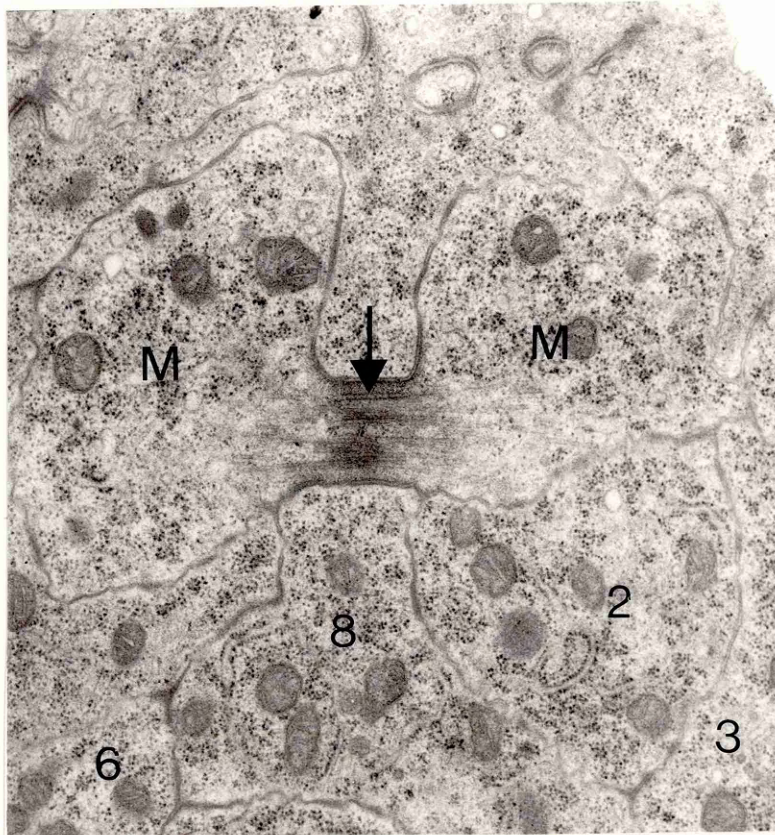


Fig. 3.21d

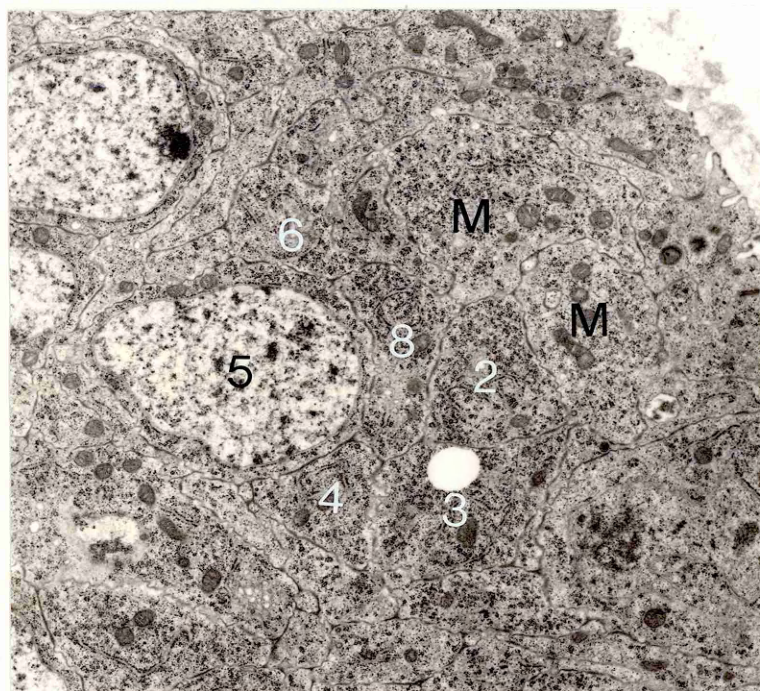


Fig. 3.21e

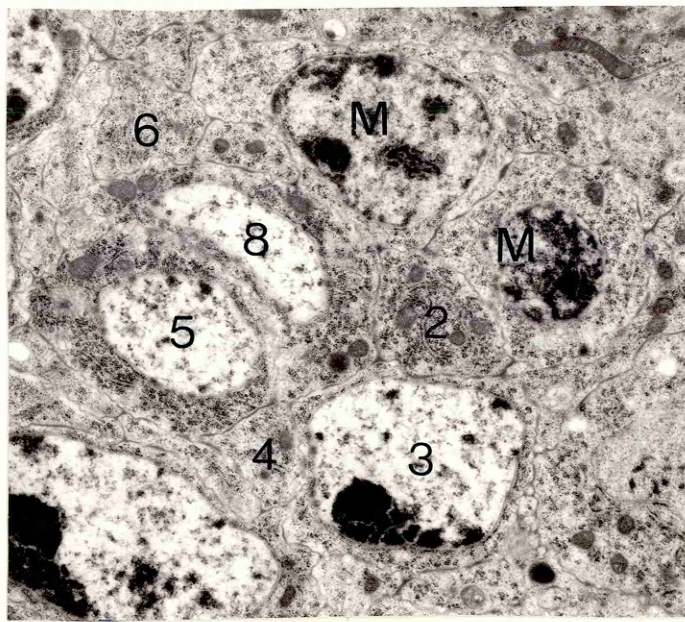


Fig. 3.21f

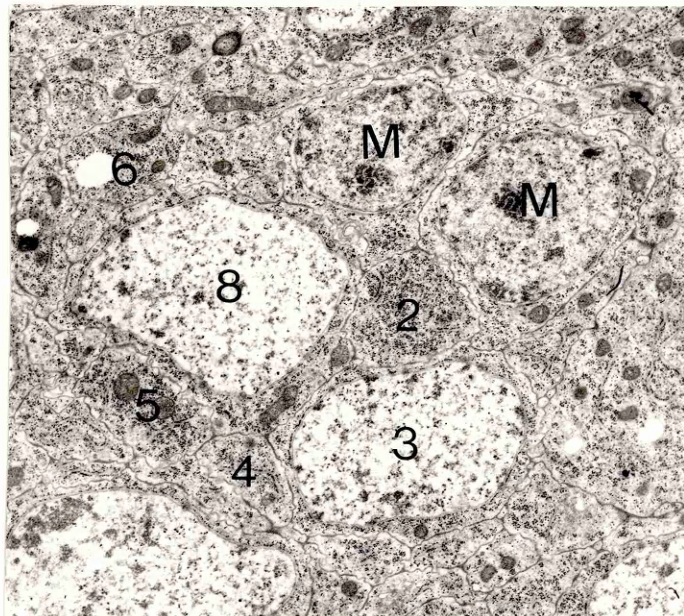


Fig. 3.21g

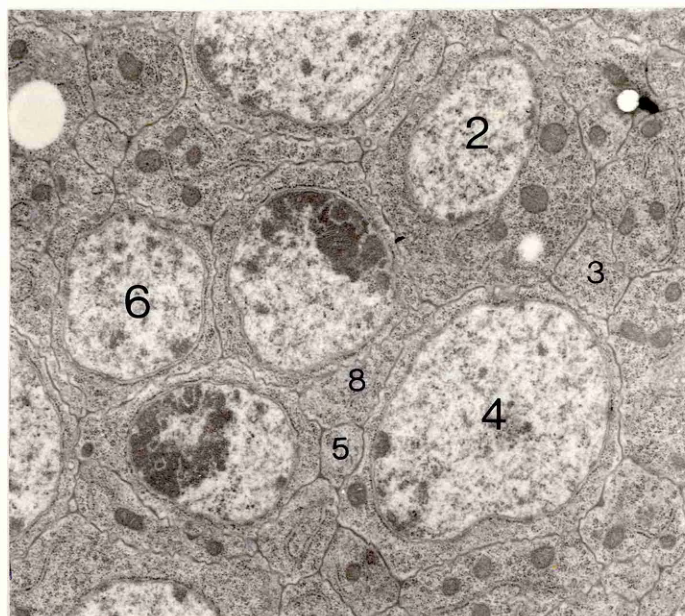


Fig. 3.21h

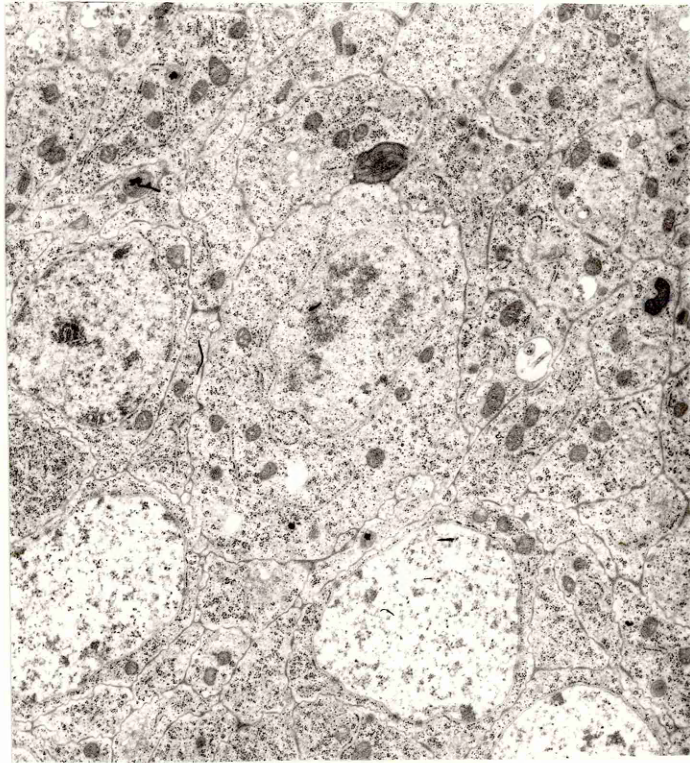


Fig. 3.21i

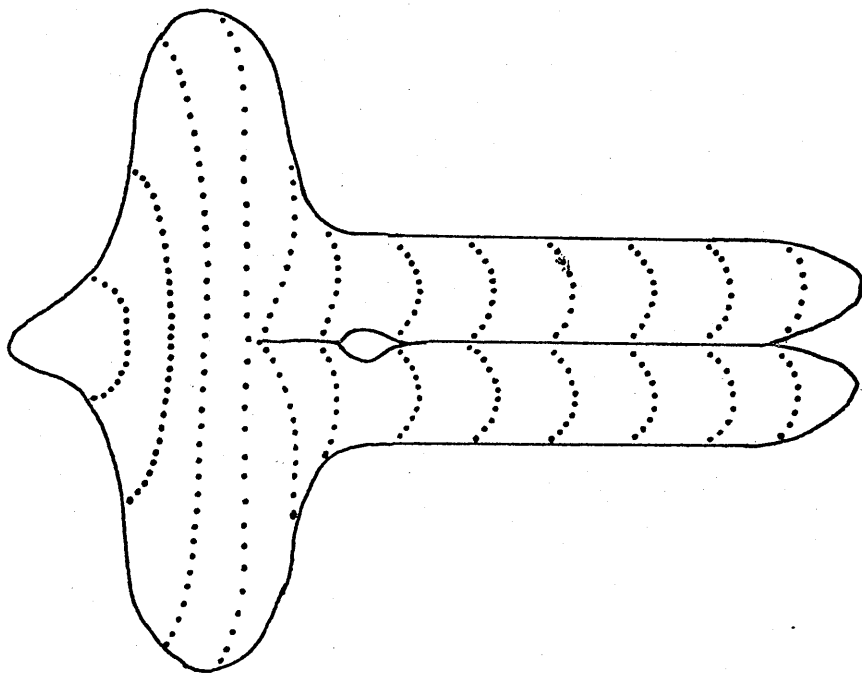
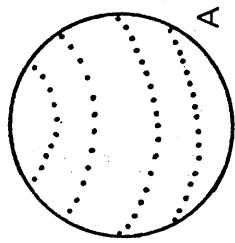
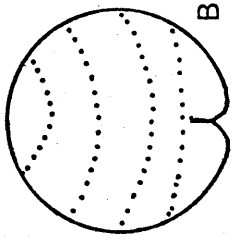


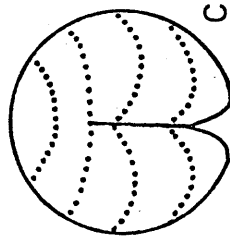
Fig. 3.22 The appearance of the mitotic cell undergoing unilateral furrowing.



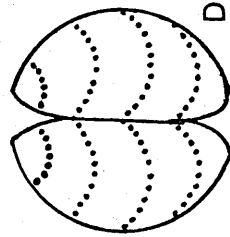
A



B



C



D

Fig. 3.23 Unilateral furrowing. The cleavage furrow first appears at one surface of the cell (B) and advances towards the antipodal surface (C) resulting in cleavage of the cell (D).

SUMMARY

The indications from the examination of the region of mitosis are that cell division is occurring in the posterior half of the MF and the pre-cluster, the independent grouping of the polar cells, earlier thought to occur prior to the second mitotic wave, only exist concomitantly with the mitotic activity, its structural existence, if at all, being ephemeral.

5. DISCUSSION

An understanding has now been achieved of the structural rearrangements occurring amongst the cells of the retinal tissue posterior to the MF. Further evidence relating to the second mitotic wave and the MF will be presented in the description of the autoradiographical studies and the process of eight cell cluster generation will be discussed later when this supplementary information can be referred to.

5.1 The Five Cell Pre-Cluster

The electron microscopic investigation has produced an understanding of the progressive development of the eight cell cluster from the most immature to the Stage III, mature, ommatidial bundle. The five cell pre-cluster has been found not to exist outside the MF and it is suspected that its location to the posterior of the MF by Ready et al, (1976) can have arisen for two reasons. Firstly, the antero-posterior limits of the MF are very difficult to establish in a section taken in the medio-lateral plane and tissue which can appear to be posterior, has, with the serial section analysis, been found to be located in the MF. Secondly, the distal appearance of the immature ommatidial bundles is that of five cell pre-clusters, the distal projections from the proximally placed equatorial cell bodies cannot easily be distinguished from those of non-receptor cells. Only when the tissue can be traced through its entire proximo-distal extent can the deceptive appearance of the distal aspect of the immature bundles be appreciated.

In the development of the retina of the greenbottle fly, Phaenicia (Melamed and Trujillo-Cenoz, 1975), no pre-clusters were detected and

analysis of axon growth indicated that the eight photoreceptor cells follow a synchronised maturation process. Eisen and Youssef (1980) in their study of retinal development of the honey bee, Apis, described the cluster formation of the nine receptor cells and no mention is made of any cluster of lesser receptor cell number, but reference is not made to whether such a situation was looked for. In hemimetabolous retinal development a small functioning compound eyes grows by, either, cellular expansion, or, addition of further ommatidia to its anterior border. In an investigation of the developing compound eye of the locust Schistocerca, where the retina enlarges by an anteriorly placed region of mitosis generating retinal cells which differentiate into ommatidia, Eley and Shelton (1976) found, posterior to the region of mitosis, ungrouped cells which gave rise to the early ommatidial clusterings - no grouping containing less than the adult complement of eight receptor cells was mentioned. In the analysis of Drosophila retinal development described in this thesis, the structural pre-cluster (as opposed to an association between the polar cells within and anterior to the MF which may not be detectable by the histological techniques used) only exists concomitantly with the second mitotic wave, its existence being only for a very short length of time. If the pre-cluster had been detected in the holometabolous and hemimetabolous examples previously described, then it could be suspected that the ephemerality of its existence in Drosophila is a peculiarity of the genus. But with the converse being the case then the pre-cluster can be considered, either, to exist in other, particularly holometabolous, insects but having a very short life span and hence would not be readily observed, or, to be peculiar to Drosophila. However, retinal development in

other holometabolous insects, particularly other dipterans, is very similar to that of Drosophila - Phaenicia (see Melamed and Trujillo-Cenoz, 1975) shows the essential retinal development characteristics of Drosophila. Hence, one might expect that the ephemeral pre-cluster would also exist in closely related species.

5.2 Specialised Cell Junctions

It appears that all the cells of the retinal epithelium of the third larval instar are connected, just below the lumen surface, by the adherens type junction (see Farquhar and Palade, 1963). The zonula adherens (ZA) type are quite evident and it is suspected that just proximal to them the macula adherens type may be present. Close scrutiny of sections taken from all levels and developmental stages has not revealed the presence of any other specialised cell junctions in the developing retinal tissue. In the examination of Phaenicia, Melamed and Trujillo-Cenoz (1975) found the zonula adherens (ZA) junctions in similar positions to those described for Drosophila, these being identified in the first, second and third larval instars. However, in their investigation of the third larval instar retinal tissue, septate desmosomes were found more proximally placed to the ZA junctions inter-relating the receptor cell membranes, and the occasional gap junction was occasionally detected. Eisen and Youseff (1980) in the examination of the developing retina of Apis found that, during the stage when ommatidial clusters were formed, the adherens type junctions were just proximal to the lumen surface, they also recognised septate and punctate junctions between the ommatidial cells. In the

investigation of retinal development of Schistocerca Eley and Shelton (1976) found, in the stage of cluster formation, that retinal cells were connected by three types of specialised junctions - septate desmosomes, punctate junctions and close junctions. Thus, by comparison with the other examples it appears that, at this developmental stage, the retina of Drosophila is somewhat lacking in specialised cell junctions. However, it must be remembered that the fixation and staining of the tissue was not done primarily to detect specialised cell junctions and it might well be the case that they are present but were not observed. However, Perry (1968a) in her investigation of pupal eye development in Drosophila only observed ZA junctions between retinal cells in the early period, septate desmosomes only occurring later on.

Sheffield and Fischman (1970) have shown that in the developing chick retina, the so called 'outer limiting membrane' is, in fact, not a membrane but a series of junctions of the ZA type, all cells projecting to the outer surface of the retina being collared and joined to their neighbours by this type of junction. Even cells undergoing mitosis were found in this condition - this being a very similar situation to that of the imaginal retina of Drosophila. Sheffield (1970) showed that the ZA junctions of the outer limiting membrane appear to hold the retinal tissue under tension, for when some of the junctions were broken following exposure to trypsin the outer limiting membrane began to curl. Sheffield and Moscona (1969) demonstrated that when the ZA junctions are broken, the cells, in the region originally associated with the junction, contracted into a 'neck'. On the cytoplasmic side of each ZA junction is an amorphous electron dense layer and Sheffield

and Fischman (1970) observed that filaments of 4-5nm diameter were anchored into this layer. Thus, it seems that the ZA junction is involved in structural and stabilising influences upon the epithelial tissue, the fibres anchoring into the junction probably being involved in cytoplasmic, and overall cellular, movements. Aubin et al, (1983) investigating changes in cell shape in fibroblasts and bone cells, have found that microfilaments, as opposed to microtubules, are responsible.

Although overt cytoplasmic differentiation, e.g. rhabdomic formation, does not occur within the retinal cells of Drosophila before pupariation, the constitution of the retinal cells of the third, larval instar, posterior to the MF, clearly shows that differential activity is taking place. However, with the only evident specialised cell junction being of the ZA type the question now arises of how this communication is achieved. Sheffield and Moscona (1970), in an analysis of clustering between disassociated chick retinal cells, where originally the only type of specialised junction present was of the adherens type, found that reaggregation of like cells seemed to be strongly associated with the ZA junctions, indicating that this type of junction may have a communicative as well as a structural function.

The similarity between the outer limiting membrane of the developing chick retina and the ZA junctions of the imaginal retinal epithelium of Drosophila is striking, and it is not unreasonable to assume that both are performing similar functions. The basement membrane and the distal 'membrane' of the ZA junctions can be viewed as providing the

skeleton upon which cellular structure and reorganisation can be supported.

5.3

Cell Death During Cytodifferentiation and Clustering of the Retinal Cells

Fristrom (1969) was able to identify only a few degenerating cells in the posterior region of the developing retina of the third larval instar, but Campos-Ortega (1980) observed a band of pycnotic nuclei posterior to the incipient eight cell clustering and suggested that cell death is an integral part of the clustering and cytodifferentiation of the retinal cells. In the extensive examination of the retinal tissue posterior to the MF described in this thesis, very few degenerating cells were observed and it can be said that cell death is unimportant in both clustering and differentiation of the retinal tissue during the third larval instar.

5.4 Cone Cells, Primary Pigment Cells, and the Hair Nerve Group

Little is known about how the primary pigment cells come to occupy their positions in the adult retinal cellular arrangement, from the cellular constitution of the late third larval instar. Shoup (1966) in her analysis of pigment granule development states that the essential adult arrangement of the pigment cells is achieved by 72 hours after pupariation. Waddington and Perry (1960) described the arrangement of the primary pigment cells in the early pupa but Perry (1968a) does not seem to be certain about this. A problem results in that both the cone cells and the primary pigment cells occupy a distal location in the adult retina and hence confusion arises when identification of the four distally placed non-receptor cell nuclei in the Stage III ommatidial bundle is attempted.

It is strongly suspected that these four cells are in fact the cone cells: they occupy approximately the expected position, all four are very similar in appearance and are symmetrically arranged about the distal tips of the retinal cells. If two of the four were, in fact, primary pigment cells then one would expect to be able to distinguish them, and also it would be sterically less complicated for two primary pigment cell-bodies to rise from the proximal pool of non-receptor cell-bodies, to their essentially adult location, than it would for two cone cells to do so. Therefore, the four distally placed non-receptor cells of the Stage III ommatidial bundle will be considered as the cone cells.

The cone cells come to occupy their distal location in two stages. Between ommatidial bundle Stages I and II, the antero-posterior pair rise, but the presence of mitosis during Stage II implies that the equatorio-polar pair which are first found distally in Stage III may not have similarly risen up. However, Campos-Ortega (1980) was able to conclude that cone, and pigment, cells are generated in the second mitotic wave. This being the case, then it can be assumed that the equatorio-polar pair of cone cells rise up the clustering between ommatidial Stages II and III as the antero-posterior pair do between Stages I and II.

Campos-Ortega (1980) identified a small amount of cell division taking place after the second mitotic wave has passed and suggested that this is due to a few secondary pigment cells arising from isolated mitoses. This mitotic activity is that detected in the region of the

Stage II ommatidial bundles, and although Campos-Ortega may well be correct in his suggestion, it should be remembered that the cells of the hair-nerve group have been totally ignored by the autoradiographical and mitotic recombination investigations of retinal cell generation during the third larval instar, and this limited mitosis could be involved in the hair nerve group production.

5.5

Cell Patterning in the Developing Ommatidial Bundles and the Influence of Steric factors

Since the only cell bodies to be found distally in the immature ommatidial bundles are those of the polar cells, then it can be concluded that the nuclei of the cells generated in the second mitotic wave move, initially, to a proximal location. The third larval instar development of the ommatidial bundles from the most immature consists basically of a rearrangement of the cell bodies of all receptor and cone cells to produce the Stage III mature ommatidial bundle. It is possible that the organisation of the cells in each bundle can be explained, to a limit extent at least, by the physical constraints that control how a group of cells can pack together. During third larval instar development posterior to the MF, an eccentric receptor cell always contacts the central (R_0) cell, the immature development leading to the situation where all eccentric receptor cells contact the central cell throughout their entire proximo-distal extent. Knowing that the equatorial receptor cell bodies initially move to a proximal location and that cell body contact with the central cell is achieved by all three after a relatively short developmental period, then it is possible to explain the behaviour of the equatorial cell bodies in terms of

competition for contact with the central cell. The cell body of R_6 is always found most distally of the three in the immature bundles, and since the polar cell bodies are found distally, then contact with the central cell is not prevented. But in IMA the cell-bodies of both R_1 and R_7 are found together at a very proximal location with that of R_1 in contact with the central cell's proximal projection, the cell body of R_7 being occluded from contact. The implications of the sequence of equatorial cell determination and generation will be discussed later, but it appears that the cell bodies of R_1 and R_7 are competing for central cell contact. Only when the cell body of R_1 has undergone a distal migration does the cell body of R_7 contact the central cell, this only occurring when the cell body of R_7 itself has migrated distally - the cell body of R_1 , as it moves distally, leaves a proximal projection about that of the central cell and hence, the cell body of R_7 can only gain contact at more distal levels where the diameter of the central cell is larger. Thus, the orderly distal migration of the equatorial nuclei, during immature development, can be explained by the need to contact the central cell coupled with the constraints imposed by steric factors.

One of the interesting aspects of cell patterning in the mature ommatidial stages is the cell pairing that occurs during Stage I and II. The cell pairing occurring within the cells R_{1-6} might be regarded simply as a packing arrangement of the cell bodies symmetrically about the equatorio-polar axis of the clustering, but the six cells that pair can be related in other ways. The eight receptor cells can be divided into three groups upon a number of criteria. Three types of photo-

sensitive pigment are found in the receptor cells - R_{1-6} contain one type and R_7 and R_8 each carry a different type (Harris et al, 1976). The mutation outer rhabdomeres absent (ora) in homozygous condition produces a phenotype in which the outer rhabdomeres (those of cells R_{1-6}) are not formed, and the mutation retinal degeneration B (rdgB) produces a similar condition where the outer rhabdomeres degenerate in adult life, in both these mutations the cells R_7 and R_8 are thought to remain unaffected (see Harris et al, 1976). Conversely the mutation sevenless (sev) in the homozygous condition produces a compound eye where the cell R_7 is absent. Occasionally cell R_8 is lacking instead, but the cells R_{1-6} remain apparently unaffected (Campos-Ortega et al, 1979; Harris et al, 1976). Hence, there is cogent evidence that within the eight receptor cells those of R_{1-6} are a sub-grouping, and although it would be difficult to ascertain whether the cell pairing, found in the mature ommatidial bundles of the third larval instar, is related to this, as will become clear later, a small amount of evidence exists in favour of it.

In the Stage I ommatidial bundle the cell bodies of R_{1-6} are arranged at three levels, distal to proximal they are R_{3+4} , R_{1+6} , and R_{2+5} , the most equatorial and polar pairs being R_{1+6} and R_{3+4} respectively. At this stage the cell body of R_8 is proximally placed in this grouping and the nucleus of cell R_7 is still undergoing its distal migration. If the cell bodies in the distal region are arranged only under the constraints of the energetics of cellular packing then it might be expected that the cell bodies of R_{1+6} should be more distally placed, e.g. at the level of cells R_{3+4} , producing a two tier system. However, the cellular

constitution of Stage I can be explained in terms of the cellular arrangement of Stage II. In Stage II all eccentric receptor cell bodies are found distal to that of R_8 , this now allows the anterior shift of this cell body which becomes evident in Stage III. If in Stage I a two tier arrangement of the cells R_{1-6} existed then the nuclei of the cone cells and cell R_7 would be prevented from coming to occupy their distal location. Thus, it appears that the arrangement of the cells R_{1-6} in Stage I is such that the two cone, and R_7 , cell nuclei can come to their distal positionings, so allowing the cone, and eccentric receptor, cell bodies to shift distally in Stage II, facilitating the anterior displacement of the central cell body. After this anterior shift by the central cell body has been performed, the eccentric receptor cell bodies are now free to move proximally, as is evident from Stage III, leaving the cone cells occupying the most distal regions. Shortly after pupariation the distal tips of the receptor cells are withdrawn (Perry, 1968a) and hence the essential, adult, retinal, cellular arrangement of the distally placed dioptric system over the photosensitive cells is being established.

For the anterior displacement of the central cell body to be affected it would be thought that, at most, only the cell bodies of $R_{1,2,3+7}$ need shift distally, cells $R_{6,5+4}$ being found to the posterior side of cell R_8 . Since all the eccentric receptor cell bodies move distally then it may be suspected that cells $R_{6,5+4}$ are each behaving as the member of a pair or as a constituent of the receptor cell sub-grouping. R_{1-6} .

The Stage III ommatidial bundle has other characteristics which can be related to the establishment of the adult cellular arrangement. When the central cell body shifts anteriorly, it moves out between cells R_1 and R_2 (its location in the adult retina) and when its distal projection is withdrawn the distal section of the prospective rhabdomeric lumen will be left. It is known that the receptor cells only separate from each other to produce the intra-receptor cell lumen, after rhabdomeric production has begun (Perry 1968a) and, hence, it can be concluded that the distal parts of the eccentric receptor cells close up when the central distal projection is withdrawn. Eisen and Youseff (1980) have described a similar lateral movement by the central (the ninth) receptor cell in Apis but here this only occurs after the initiation of rhabdomere production and the displacement proceeding in a disto-proximal sequence.

The cell-body of R_4 in the distal half of the Stage III ommatidial bundle is displaced from contact with the distal projection of the central cell and again this is thought to be part of the cellular movements, which, during the pupal stage, will give rise to the adult arrangement of the retinal cells.

5.5

The region of the head disc occupied by retinal tissue

Sprey and Oldenhave (1974) have challenged the fate map of the head disc of Drosophila produced by Ouweneel (1970). They argue that the peripodial membrane, previously ignored, gives rise to a sizeable portion of the head cuticle (a finding supported by Milner et al, (in proof)), and produced a tentative fate map (see Fig. 1.8). The

detailed electron microscopical analysis of the retinal tissue described in this thesis has revealed that the field of developing ommatidial bundles extends posteriorly and laterally right up to the edge of the epithelium where contact with the peripodial membrane is achieved. This finding supports the tentative fate map produced by Sprey and Oldenhave (1974), and conflicts with that of Ouweneel (1970) who proposed that the retinal field was surrounded on all sides by prospective head cuticular tissues.

CHAPTER 4. AUTORADIOGRAPHICAL STUDIES

1. STUDIES OF THE MITOTIC WAVES USING ^3H THYMIDINE

The electron microscopical studies have indicated that the second mitotic wave occurs in a position anterior to that described by Ready et al, (1976), being located in the posterior region of the MF. As has been previously explained, the extent of the MF is difficult to establish in sections taken in the medio-lateral plane and accordingly it was decided to study the second mitotic wave by autoradiography of sections taken in the proximo-distal plane. Although the major aim of the autoradiographical studies was to establish the topographical position of the second mitotic wave, it was recognised that the appearance of the anterior mitotic wave may well cast light upon the nature of retinal tissue generation i.e. whether a wave of proliferation traverses a pre-existing retinal field or whether retinal cells are generated by an anteriorly positioned zone of mitosis (see Chapter 1, 3.1.4).

1.1 In vitro labelling with ^3H thymidine

The electronmicroscopical studies have shown that the immature ommatidial bundles have only the polar cell nuclei located distally. Hence, nuclei generated by the second mitotic wave must move initially to a proximal position. Knowing that in the MF all cell-bodies are found proximally placed it was suspected that if labelling was performed in vivo then confusion would result from rising and falling label occurring in a relatively small (approximately two ommatidial rows) area of tissue. Labelling of mitotic cells for a short period

followed by culturing in vitro, was thought might ameliorate this problem assuming that cell division could be supported for a sufficient length of time. Bullmore (1977) performed a detailed analysis of cell division in third larval instar imaginal discs (leg and wing) in vitro, and found that in Shields and Sang Medium mitosis fell during a five hour period to a relatively low activity. Campos-Ortega and Hofbauer (1977) recorded that the second mitotic wave advances at approximately one ommatidial row every two hours. Coupling these two pieces of information it was decided that it was feasible to perform the experiment in vitro. The head disc is peculiar in that differentiation begins before the pupal stage and it is suspected that retinal differentiation may be initiated and maintained by the increasing level of β ecdysone (Martin Milner, private communication). Bullmore (1977) has shown that mitosis is maintained, in vitro, at a higher level in the presence of a low level of β ecdysone than in its absence. The in vitro culturings of the discs were performed both with and without a low level of β ecdysone in the medium.

1.1.1 Results

Fig. 4.1 shows the appearance of the label after culturing for thirty minutes with ^3H thymidine followed by culturing for differing lengths of time, in medium alone, before fixation. The autoradiographs displayed are all derived from culturing in the presence of β ecdysone, but the appearance of the label after culturing for equivalent periods without the presence of the hormone was not appreciably different. All times refer to the total culturing duration i.e. the sum of labelling, and post-labelling, culturing. Examination of the autoradiographs revealed that in each disc label was found at differing positions on

different sections. The autoradiographs presented have been chosen because they indicate the range of spread of the label found for each culturing period.

After one hour's culturing the label of the second mitotic wave is found proximally, centering in the MF (Fig. 4.1a), and after two hours the label has moved into a more posterior position and is showing a more distal spread (Fig. 4.1b). After four hours the label has continued with its posterior and distal movement (Fig. 4.1c) but autoradiographs of sections from discs cultured for six hours (Fig. 4.1d), although showing the label to be slightly more posteriorly and distally placed, could not be readily distinguished from those of four hours.

Examination of Fig. 4.1 reveals that, although the label does show a distal movement over the culturing periods, it is never found close to the distal surface. It has been reported that in imaginal discs a dividing cell detaches from the basement membrane and rounds up at the lumen surface (Ready et al, 1976; Poodry and Schneiderman, 1970), and the electron microscopic studies have revealed mitotic cells of the second wave in the distal region of the disc. Accordingly, the validity of the results derived from the in vitro culturing must be questioned. The failure to observe the label of the second mitotic wave close to the distal surface can be explained by three possibilities. Firstly, the medium may have failed to support mitosis, those cells in S-phase uptaking the labelled thymidine but failing to undergo the later stages of the division cycle. However, examination of the autoradiographs where the anterior mitotic wave was present revealed that label here

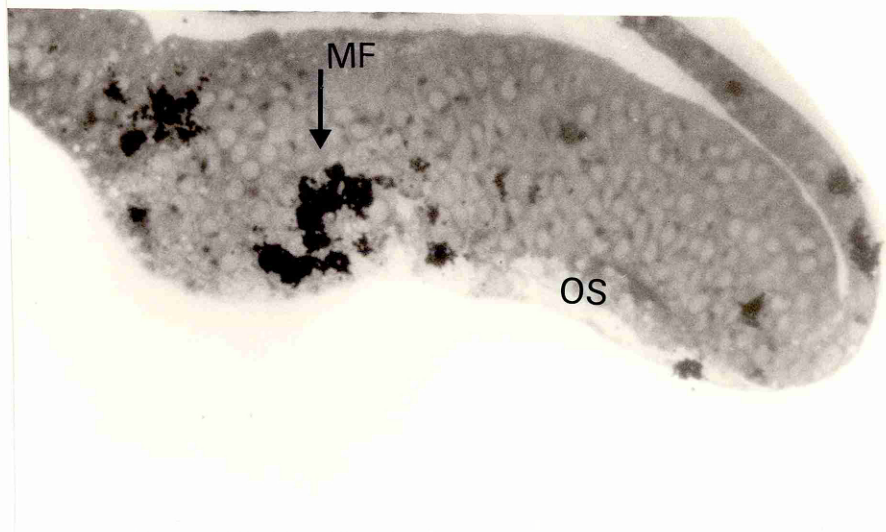


Fig. 4.1a Autoradiograph of disc labelled in vitro with ^3H thymidine, cultured for 1 hour. Arrow: second mitotic wave; MF: morphogenetic furrow; OP: optic stalk.

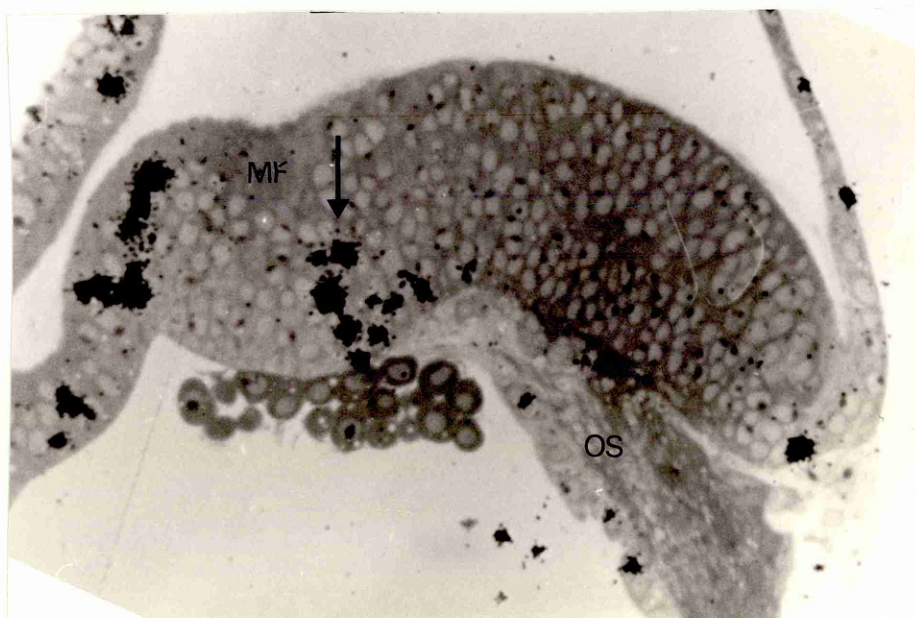


Fig. 4.1b Autoradiograph of disc labelled in vitro with ^3H thymidine, cultured for 2 hours. Arrow: second mitotic wave; MF: morphogenetic furrow; OP: optic stalk.

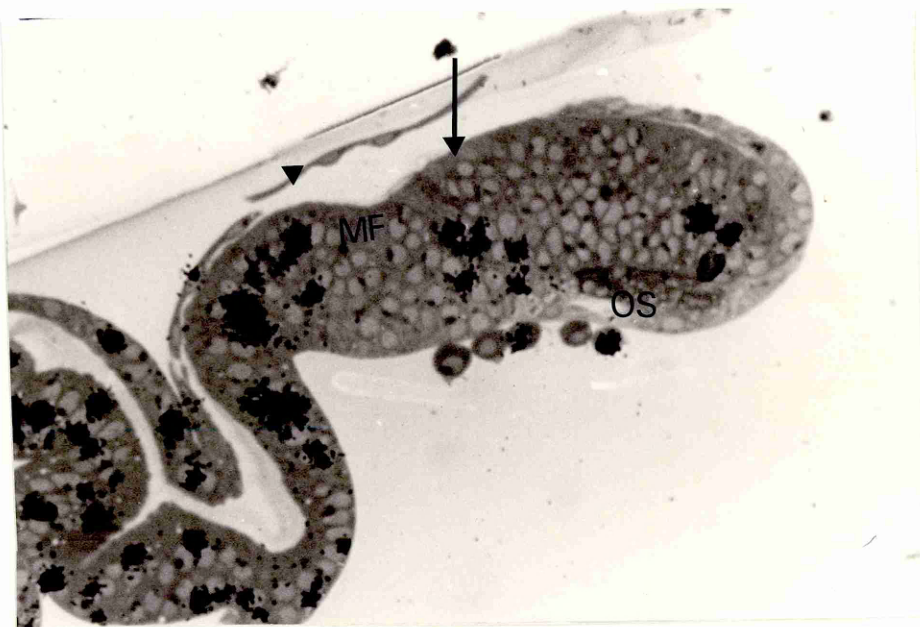


Fig. 4.1c Autoradiograph of disc labelled in vitro with ^3H thymidine, cultured for 4 hours. Arrow: second mitotic wave; Arrowhead: anterior mitotic wave; MF: morphogenetic furrow; OP: optic stalk.

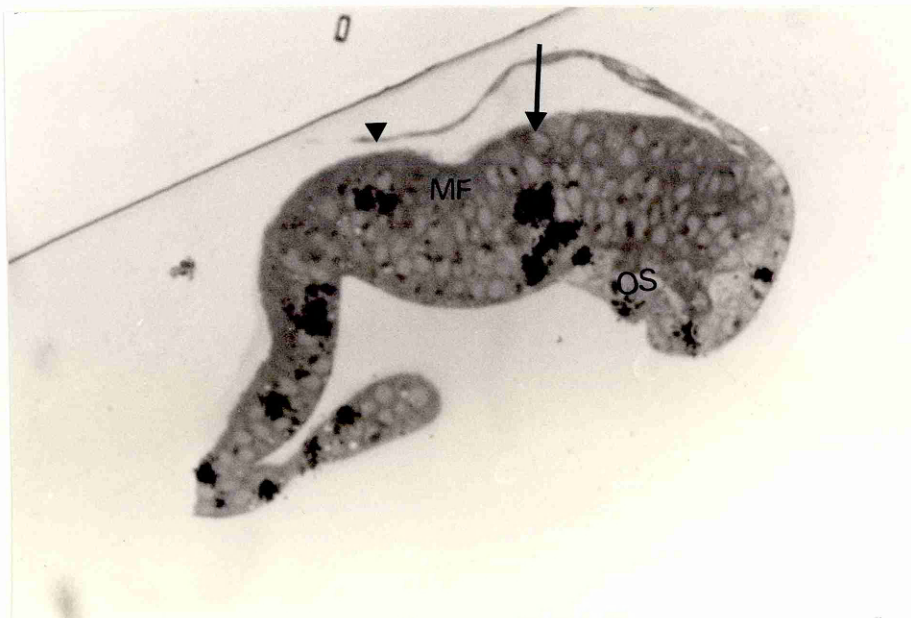


Fig. 4.1d Autoradiograph of disc labelled in vitro with ^3H thymidine, cultured for 6 hours. Arrow: second mitotic wave; Arrowhead: anterior mitotic wave; MF: morphogenetic furrow; OP: optic stalk.

was regularly found close to the distal surface (Fig. 4.1c). Secondly, the mitosis of the second wave may have been supported by the medium but the experimental techniques adopted failed to detect it. This could result from either, the movement to the distal surface and later proximal fall being rapid and occurring between the staged culturing periods, or, the label was taken up by cells destined to divide more than six hours afterwards. Thirdly, failure of the label to rise into the more distal regions of the disc may be accurately indicating the movements of the chromosomal material in the second mitotic wave. This, although conflicting with the knowledge that mitotic cells are found in the distal regions of the disc, may, when taken with the knowledge that a unilateral furrowing mechanism operates, be the case, and this will be dealt with in greater detail in the Discussion.

1.2 In vivo labelling with ^3H thymidine

The examination of the second mitotic wave using in vitro techniques failed to produce clear indications of where in the developing retinal field this mitosis occurs - the label close to the distal surface being thought necessary to accurately determine its location. Following Ready et al, (1976) the labelling was now performed in vivo.

1.2.1 Results

When larvae were fixed four hours after being injected with ^3H thymidine the label was regularly found close to the distal surface of the disc. Six head discs from separate larvae were examined and all were found to show the label reaching the distal regions of the disc at a similar position. Those discs taken from larvae which were

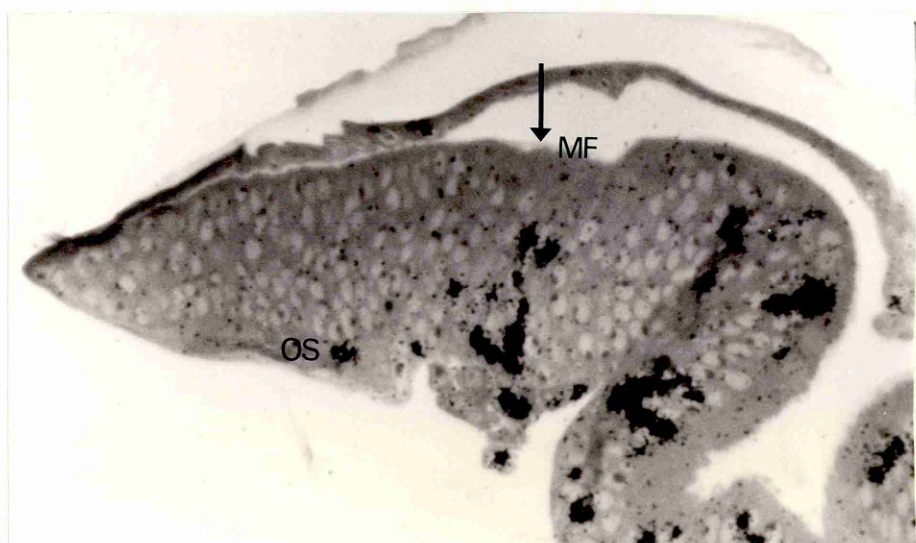
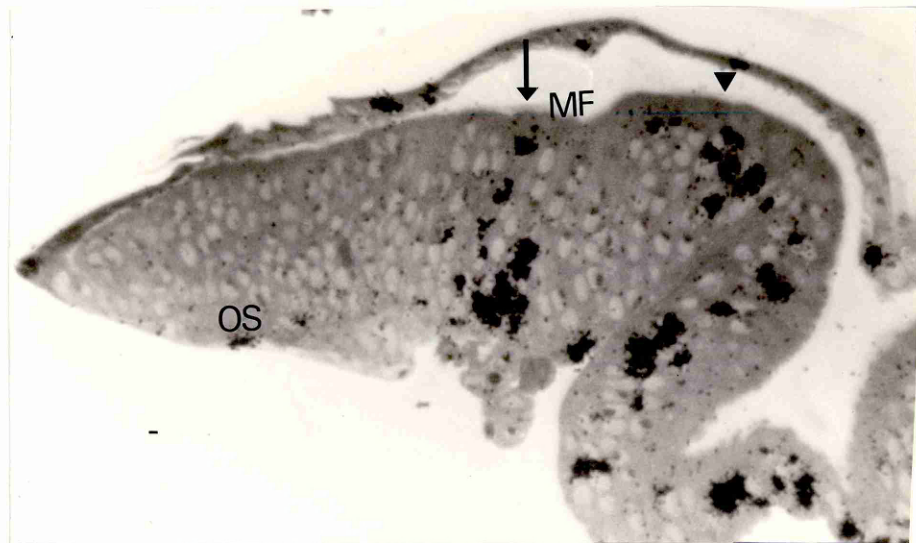
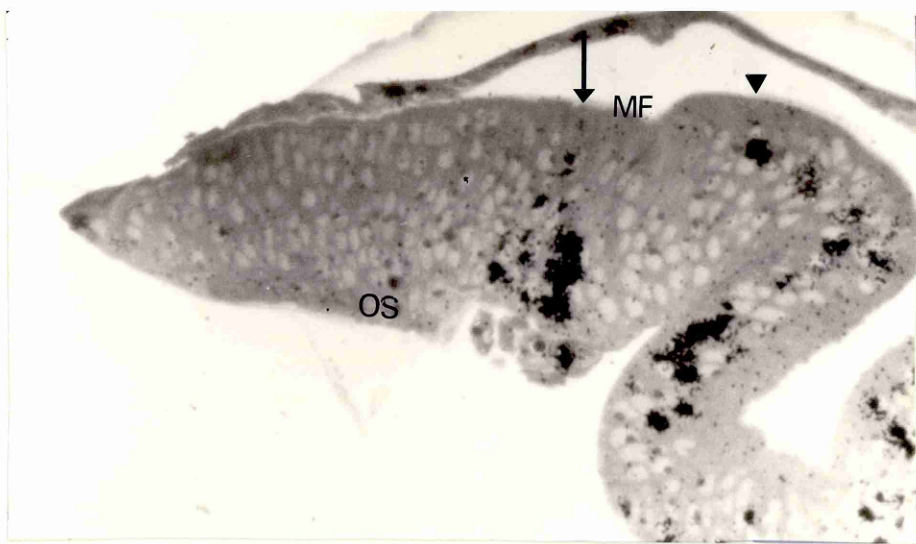


Fig. 4.2 Autoradiographs of sections from disc fixed 4 hours after labelling in vivo with ³H thymidine. Arrow: second mitotic wave; Arrowhead: anterior mitotic wave; MF: morphogenetic furrow; OP: optic stalk.

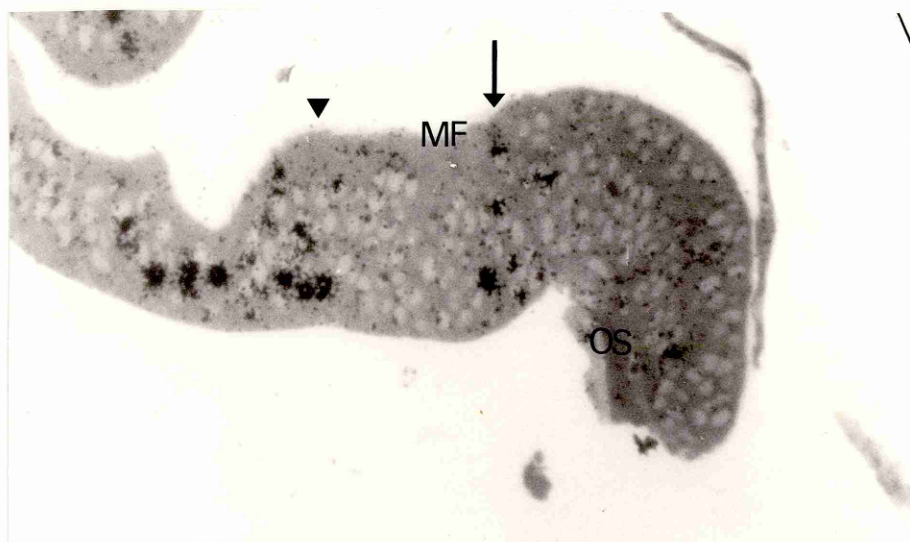
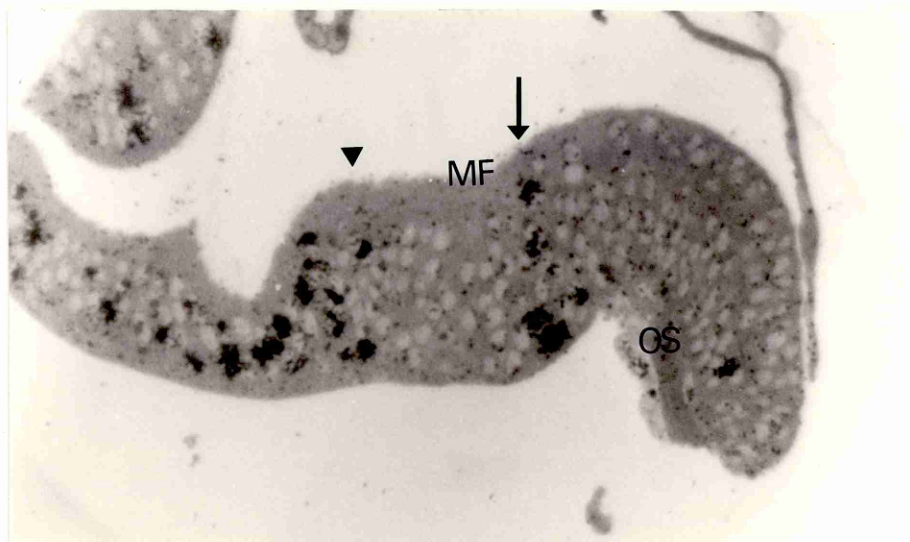


Fig. 4.3 Autoradiographs of sections from disc fixed 4 hours after labelling in vivo with ³H thymidine. Arrow: second mitotic wave; Arrowhead: anterior mitotic wave; MF: morphogenetic furrow; OS: optic stalk.

allowed two and six hours development after injection showed a similar labelling appearance to their in vitro cultured counterparts.

Figs. 4.2 and 4.3 show the typical appearance of the second mitotic wave as the dividing cells are rising into the distal region of the disc. It can be seen that the cell division is taking place in the posterior region of the MF.

1.3 Examination of the anterior mitotic wave

When the anterior mitotic wave is present in a disc then it is regularly found in the most anterior regions of the apparent developing retinal field (Figs. 4.1c,d and 4.2). Label was found to be displaced from the most anterior regions but this was always in a disc where most sections displayed label at the anterior limit of the retinal field.

2. A STUDY OF RNA SYNTHESIS IN THE THIRD LARVAL INSTAR RETINAL FIELD

Poodry et al, (1973) using the temperature sensitive mutation shibire, administered heat shock pulses to third instar larvae of various ages and elicited a band of disruption in the dorso-ventral axis of the adult retina. The anterior displacement of the disruption was directly related to the age of the subject larva. Campos-Ortega and Hofbauer (1977) traced the movement of the mitotic waves in the retina during the third larval instar and that of the second wave correlates strongly with the anterior displacement of the induced band of disruption described. The studies in this thesis have revealed that the second mitotic wave is associated with the MF and it can be

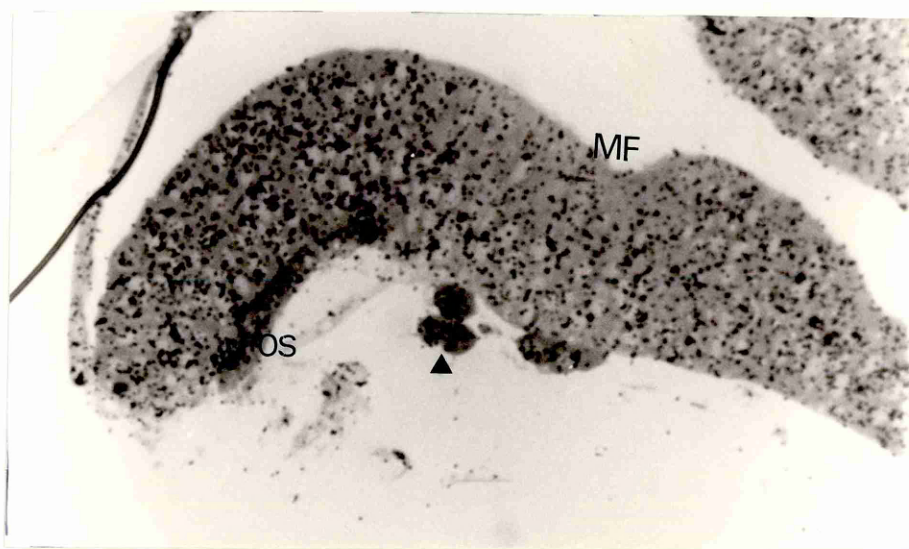


Fig. 4.4 Autoradiograph of disc labelled in vitro with ^3H uridine for 30 minutes. Arrowhead: macrophage-like cells; MF: morphogenetic furrow; OP: optic stalk.

inferred that the target tissue of the heat shock pulses is in, or around, the MF. van Breugel et al, (1975) were able to elicit, by injection of third instar larvae with actinomycin D and fluorouracil (which inhibit RNA transcription and translation respectively), similar bands of disruption in the adult retina - their interpretation was that cell differentiation was being disrupted. Since RNA synthesis is required for differentiation to be achieved then it was considered that an investigation of RNA synthesis in the retinal field during the third larval instar might indicate where the major differentiation was occurring.

Third larval instar head discs were cultured in vitro for 30 minutes with ^3H uridine and fixed immediately afterwards.

2.1 Results

Many head discs were examined autoradiographically and Fig. 4.4 shows their typical appearance. It can be seen that heavy labelling is found in the posterior regions of the developing retina where the mature ommatidial bundles are found. The most intense labelling is found in the developing optic stalk. In the regions anterior to the mature ommatidial bundles less RNA synthesis is indicated, the label anterior to the MF being somewhat heavier than within and just posterior to it. It is interesting to note that the macrophage-like cells described by Ready et al, (1976), which are located under the basement membrane are also showing a considerable amount of RNA synthesis.

3. DISCUSSION

3.1 The location of the second mitotic wave

The electron microscopic investigations revealed that the second mitotic wave occurs in the posterior region of the MF and the autoradiographical studies have provided confirmatory evidence. This result apparently conflicts with the findings of Ready et al, (1976), but preliminary autoradiographical studies performed by the present author revealed that in the medio-lateral plane the second mitotic wave does appear to be posteriorly displaced from the MF. This anomaly can be explained by the appearance and structure of the MF. Firstly, as has previously been explained, the appearance of the MF in the medio-lateral plane is deceptive. The indentations in the lateral wings of sections indicate the centre of the MF but it extends more posteriorly and anteriorly than is evident. Secondly, within the MF all cell bodies are found proximally placed. Outside the MF the nuclei are arranged at differing levels within the proximo-distal axis of the disc, and hence in the anterior and posterior regions of the MF the cells are found to bow to accommodate the packing arrangement of the cell bodies in the centre (Fig. 4.5). As can be seen from the autoradiographical studies the main 'pool' of label is found proximally placed and owing to the fact that cells in this region are bowing, the label is present within cells of the MF but appears to be more posteriorly positioned. Thus the apparent posterior displacement of the label from the MF in the medio-lateral plane results from the MF extending more posteriorly than is evident, coupled with the posterior positioning of the cell bodies in the posterior aspect of the MF (Fig. 4.5).

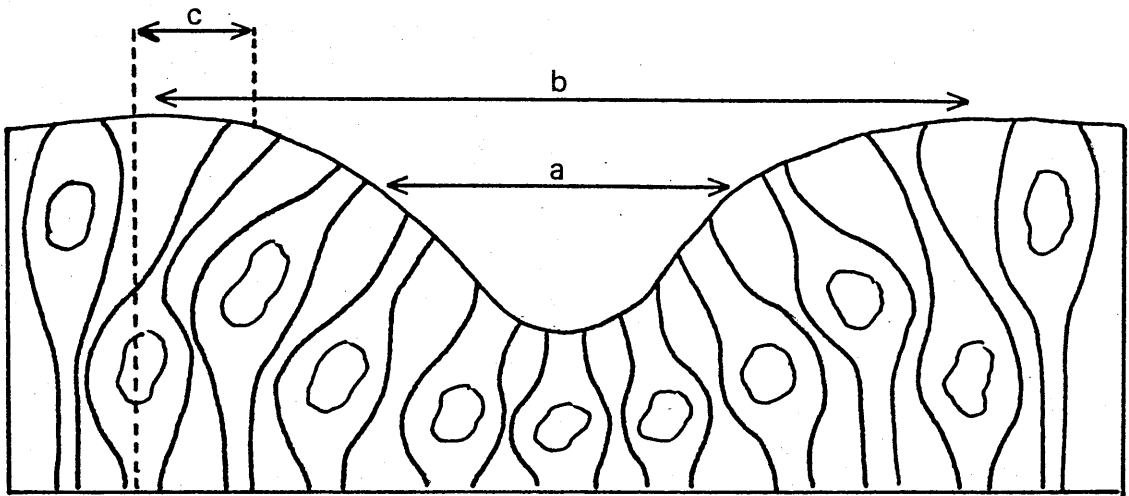


Fig. 4.5 The appearance of the morphogenetic furrow in the proximo-distal plane. The extent of the furrow (b) is greater than apparent in the medio-lateral axis (this being indicated by (a)). The cell-bodies in the posterior region are displaced posteriorly in relation to their distal parts (c).

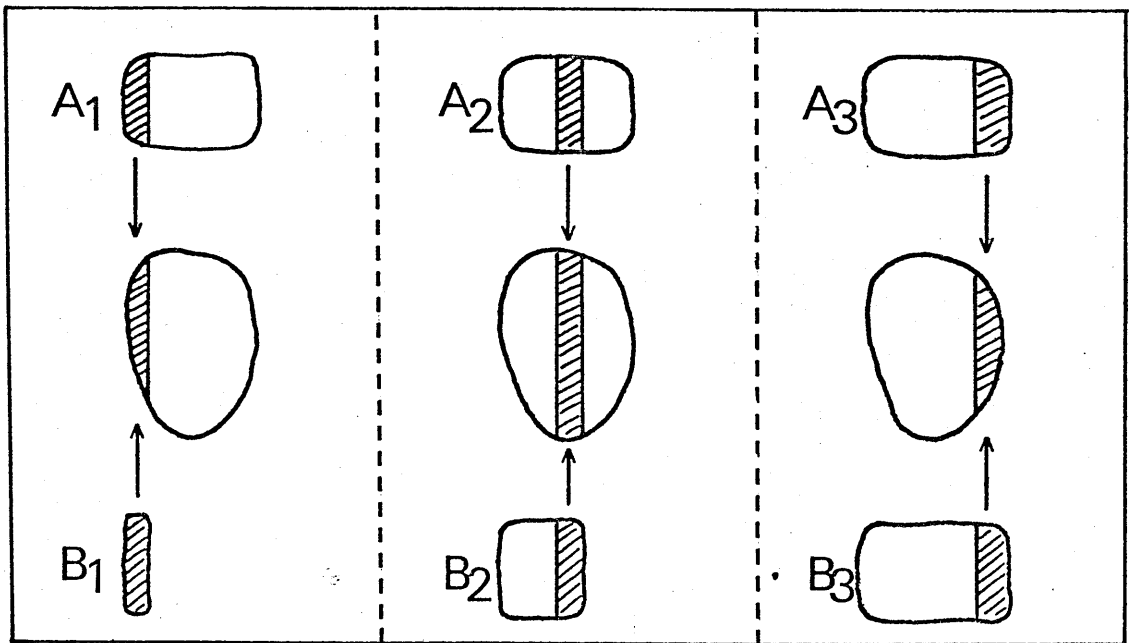


Fig. 4.6 A wave of mitosis traversing a retinal field (A) and an advancing eye margin (B) labelled at times 1, 2 and 3 will produce the same patterning of label in the adult retina.

3.2 The unilateral furrowing mechanism

As has been described in the electron microscopical studies a unilateral furrowing mechanism has been detected operating in the cell divisions of the second mitotic wave. Using the electron microscope, the second mitotic wave has been examined in many head discs and unilateral furrowing has regularly been found. The more usual furrowing mechanism, where the furrow appears at the same time in the entire equatorial region of the dividing cell, has not been observed, but it is thought that owing to its appearance this form of furrowing will not be as readily detectable.

In animal cells, two mechanisms for furrow establishment are known to exist. The more typical form is where the equatorial surface of the cell is directly and simultaneously altered so that furrowing appears around the entire equatorial region, and the organelle responsible, the contractile ring, has been likened to a purse string (see Schroeder, 1975). This type of furrowing occurs when a large mitotic spindle is found centrally placed within the cell. But when the mitotic apparatus is somewhat eccentric, furrowing first begins in the nearest surface and later in the more distant surface (Dan and Dan, 1947). The mitotic apparatus is known to be responsible for establishing the furrow (Rappaport and Rappaport, 1974) and in the situation where the spindle is somewhat eccentrically placed the entire furrow results from stimulation by the mitotic apparatus, the delay in its appearance in the more distant surface being thought due to the distance the stimulus needs to travel (see Rappaport, 1975). Thus, when the spindle is somewhat eccentrically placed the same form of contractile ring is operating

as in the typical 'purse string' form. But when the mitotic apparatus is highly eccentrically placed within the cell, a second, different, furrowing mechanism is established, this being termed unilateral furrowing. In unilateral furrowing, the highly eccentrically placed mitotic apparatus stimulates the furrowing in the nearby surface. The antipodal surface is thought too distant for the stimulus to reach and the furrow once established in the nearby surface appears to be self propagating (Rappaport, 1975; Rappaport and Conrad, 1963). Hence, the furrowing first appears in one side of the cell and progressively moves towards the other (Fig. 3.23). The furrowing described in the second mitotic wave is the unilateral form, beginning proximally and progressing distally.

A literature search performed by the present author indicates that this is the first description of the unilateral furrowing mechanism in Drosophila - a finding supported by Richard Warn (private communication). The only example of a similar process in Drosophila is that by which cellularisation of the blastoderm is achieved. This is effected by an elaborate contractile ring system which links up over the embryo and results in the cells which form the middle of the embryo being significantly larger than those at either end (Warn and Magrath, in press). It is known that cleavage occurs more rapidly on the ventral surface (Mahowald, 1963) and coupling these facts together it seems likely that the contractile ring network contracts faster in one region (see Warn and Magrath, in press). Thus, it appears that a kind of unilateral furrowing operates during the cellularisation of the blastoderm. It should be stressed however that the unilateral furrowing in the blasto-

derm is producing cells of differing sizes, but the division of the second mitotic wave is symmetrical, cleaving the cells in the proximo-distal/lateral axes producing two equally sized cells. Where asymmetrical division is found in Drosophila, unilateral furrowing is not found to operate.

The peculiarity of the unilateral furrowing to the second mitotic wave in the retinal differentiation of Drosophila leads to the question of whether it is an important aspect in the generation of the eight cell clusters of receptor cells. There is little evidence to provide the answer, and accordingly only tentative proposals can be made. Perhaps the most simple explanation may be that the unilateral furrowing mechanism results directly from a rapid division process. Ready et al, (1976) for the eye, and Poodry and Schneiderman (1970) for the leg, have described the dividing cells in the imaginal epithelium to lose contact with the basement membrane, round in the distal region, divide and then extend back down to the basement membrane. All cells in the MF have their nuclei proximally placed, and if mitosis is occurring rapidly then telophase might well be reached before the rounding up process is complete. Since the nuclei are initially found proximally in the cell and if the mitosis is occurring rapidly then the spindle may be expected to form in a proximal location and hence result in a unilateral furrowing being initiated proximally.

Three points need to be mentioned here. Firstly, all the evidence relating to unilateral furrowing indicates that for it to operate as observed in the second mitotic wave, the mitotic apparatus must be

located highly proximally. Secondly, mitotic figures have not been detected proximally in the tissue when examined with the electron microscope, but an extensive search was not carried out, and, suspecting the mitosis to be rapid, then only few would be present. Thirdly, the cell undergoing unilateral furrowing has two inchoate daughters bearing very proximally the two incipient nuclei (see 4.1.3). This can be explained by the nuclei, persisting at the locations the poles of the spindle occupied, or by them moving proximally in the 'feet' being put down to the basement membrane. Since cytokinesis is not complete then this is consistent with a rapid division process.

3.3 The behaviour of the cells of the second mitotic wave

The autoradiographical studies have shown that label does rise to near the distal surface of the disc, indicating that the mitotic apparatus is in this region. However, it need not be that all label rises to this distal level. There is difficulty in distinguishing rising and falling label. Knowing that chromosomal replication is semi-conservative then a post-mitotic cell should carry half the amount of label of a pre-mitotic cell and this provides a key for identification. But there are many complications which makes this identification difficult. Firstly, label uptake is during S-phase and when the label is supplied, some cells will be just entering, others will be in the middle of, and some will be towards the end of, DNA synthesis, and accordingly differential labelling of the cells is expected. Secondly, the amount of label overlying a cell in an autoradiograph is dependant upon the amount of chromosomal material in the sections and as a result sections through the centre of a nucleus will show more label than those through the more peripheral regions. Thirdly, the second mitotic wave is known

to be restricted to about two ommatidial rows, and since the equatorial receptor cells, the pigment cells, and the cone cells are known to be generated by this mitosis (Ready et al, 1976; Campos-Ortega, 1980), then thirteen cells* are produced per ommatidium. The presence of so many mitotic cells within a confined area of tissue results in confusion. Coupling all these facts together it can be said that mitosis occurring below the distal region of the disc cannot be assumed to be indicated by the autoradiographs. However, there is secondary evidence indicating that this may well be the case. Examination of the discs where the label is reaching the distal regions of the disc has revealed that although label is often found distally it is not occurring at the frequency expected. This is based on the following argument. Thirteen cells per ommatidium are thought to be generated by the second mitotic wave (see above). Lawrence and Green (1979) performed a clonal analysis of the later cell lineages of the retinal cells in Drosophila. Irradiation of larvae 4± hours before puparium formation (when the first mitotic wave has ceased) produced fourteen clones, three being two cells and the remainder being single cells. The indication of this is that the cells of the second mitotic wave only undergo a single mitosis (a finding supported by Hanson, unpublished - see Kankel et al, 1980). Campos-Ortega and Hofbauer (1977) have shown that the second mitotic wave advances at a rate of one ommatidial row per two hours. With the knowledge that approximately seven mitoses are expected per ommatidium,

* 3 equatorial receptor cells, 4 cone cells, 2 primary pigment cells, 3 secondary pigment cells, and one tertiary pigment cell. (see Chapter 1, part 1).

then, if all divisions were taking place distally, it would be thought that at the stage where the mitosis is occurring that label should be persistently found in the distal regions. This is not the case: only 4-5 patches of label are found distally per disc and hence this may well indicate that the division is not necessarily always occurring in the most distal regions of the disc.

If this is the case then it might explain the failure of the label to be found in the most distal regions of the discs cultured in vitro. The experiment was performed with the knowledge that the mitotic rate would fall over the incubation period to approximately $1/6$ of the normal rate (see Bullmore, 1977). But since the goal of the experiment was to detect the location of the second mitotic wave, this lowered rate of mitosis was considered sufficient. Hence it might be thought that the distal and posterior movement displayed by the label over the culturing periods may well be resulting from the more proximally positioned mitoses, the divisions occurring at the distal surface not being detected owing to the low mitotic rate. However, with the knowledge of the reduced mitotic rate it is quite possible that a sufficiently long culturing period was not allowed for the divisions to occur.

3.4 The mode of retinal tissue generation

In hemimetabolous retinal development a small functioning compound eye grows, either, by expansion of the existing cells, or, by addition of retinal cells to its anterior from an anterior zone of mitosis. In the cockroach, where retinal growth is by the latter mechanism, it has been shown that the additional retinal cells are produced by a proliferating zone lying just within the anterior eye margin. The earlier

theory that cells are recruited into the retina from the head capsule epidermis (Hyde, 1972) being discounted (Nowel and Shelton, 1980).

In holometabolous retinal development a proliferative division (the anterior mitotic wave) has been described traversing a pre-existing retinal field (Ready et al, 1976; Campos-Ortega and Hofbauer, 1977).

It has been suggested by Nowel and Shelton (1980) that both holometabolic and hemimetabolic retinal development may be occurring in a similar manner i.e. that retinal tissue is generated by a proliferating zone lying within the anterior eye margin. Ready et al, (1976); Campos-Ortega and Hofbauer (1977), and Campos-Ortega and Gateff (1976), after labelling the proliferative division during the third larval instar found that in the adult retina the label shifted from posterior to anterior with the increasing age of the subject larva. These results were interpreted as a wave of proliferation passing across a prospective eye field.

However, as proposed by Nowel and Shelton (1980), these results would also be expected if an advancing eye margin* was operating (Fig. 4.6).

Examination of the anterior wave autoradiographically has revealed that when present it was always found to extend, in most sections at least, to the anterior edge of the retinal field. Early third instar eye discs were also examined to ensure that the anterior positioning of

* The term advancing eye margin has been used as a convenient description of the operation of the proliferative zone in the anterior eye margin. The movement of the proliferative zone is in relation to the retinal tissue and therefore this system can also be considered as a stationary meristem generating retinal tissue to its posterior.

the first mitotic wave was not due to it having already traversed the retinal field. This observation tends to favour the advancing eye margin theory. However, the appearance of the anterior retinal field may be deceptive, it may only appear as such once the wave of proliferation has reached that position. Meinertzhagen (1973) describes the thickening of an epithelium into an optic placode by mitotic activity.

There is evidence to support the advancing eye margin theory. Becker (1957) performed a clonal analysis of retinal development in Drosophila and found that clones induced during the second larval instar always spread to the anterior margin of the adult retina - a finding supported by Campos-Ortega and Waitz (1978) using M^+ clones. If a wave of mitosis was passing over a pre-existing retinal field then clones would not necessarily be expected to always spread to the anterior of the retina.

Differentiation proceeding from posterior to anterior has been described for the retinal development of many holometabolous insects - Wolsky (1956) for the moth Bombyx; White (1961) for the mosquito Aedes, and Umbach (1934) for Ephestia. Wolsky (1956) has described that cautery of Bombyx during pupation produces differing results. If, at the beginning of pupation the back of the eye disc was cauterised, then retinal development failed. But cautery at the same place later in pupation resulted in the rear eye field being affected, while treatment of the anterior of the eye disc early on had no effect, but later on prevented further eye development. This and similar experiments have led to the view that retinal development spreads anteriorly from a

posteriorly positioned differentiation centre (see Meinertzhagen, 1973). If a wave of mitosis passes across the prospective retinal field then the cautery of the anterior field early in development would still be expected to produce disruption in the anterior adult retina. But if the advancing eye margin operates, then eye development will only be affected if the proliferative zone itself is destroyed, and the results described are consistent with this.

Campos-Ortega and Gateff (1976) have shown that the head disc of a late second larval instar is able to differentiate a small disrupted retina containing receptor, pigment, and cone, cells. As has been described earlier, pigment and cone cells are known to be derived from the second mitotic wave and hence, the tissue able to generate a small retina might be considered analogous to the small functioning compound eye found in hemimetabolous development. This can then be viewed as indicating a similar retinal developmental process in both the endopterygotes and the exopterygotes.

Experiments performed on the developing retina of Ephestia by Nardi (1977) demonstrated that tissue found anterior to the prospective retina when transposed to the ommatidial 'field' produced retinal tissue. This result indicates that prospective cuticular cells can be recruited into the retina. However, the ability of the cuticular cells to differentiate retina diminished with their distance from the differentiation centre (see above). Egelhaaf et al, (1975), again investigating retinal development in Ephestia, observed that the eye anlage incorporates adjacent epidermal material.

Retinal growth in Ephestia consists of a bidirectional expansion from the differentiation centre along the antero-posterior axis, whereas in Drosophila retinal growth is a unidirectional anterior expansion (Nardi, 1977).

The bidirectional growth of the retina of Ephestia is reflected in the presence of two dorso-ventral furrows that can be distinguished at the anterior and posterior boundaries of the differentiation centre, these furrows being regarded as equivalent to the morphogenetic furrow of Drosophila (Nardi, 1977). In comparing the anterior development of the retina of Ephestia with the retinal development of Drosophila, Nardi (1977) interpreted the behaviour of both as indicating that the same form of recruitment process occurs. He postulated that the wedge shape clones found by Becker (1957) (see above) were resulting from an advancing differentiation centre incorporating new cellular elements into the developing retina. However if recruitment does occur in the retina of Drosophila then it would not be expected that the clines induced by Becker (1957) would always extend to the anterior edge of the retina as they do.

Although there is much evidence to indicate that an advancing eye margin operates in holometabolous retinal development, the investigations into retinal development in Ephestia indicate that this may not necessarily be the case, and cell recruitment onto an advancing differentiation centre may well operate.

3.5 Differentiation and determination of the retinal cells

The electron microscopic studies have shown that the last receptor cell to project an axon into the developing optic stalk is R_7 and from this it can be inferred that this is also the last to begin differentiation. Cell R_6 , the one found persistently to be the most distal of the equatorial receptor cells in the immature ommatidial bundles has been detected projecting into the optic stalk when cell R_1 does not. It is suggested that of the equatorial receptor cells R_6 is the first, and R_7 the last, to begin differentiation. This may be indicative of the sequence of determination of these cells. If this is the case then the equatorial receptor cells may be taken up sequentially into the developing ommatidial cluster. However only slight evidence exists to support this. In the pre-cluster described (Chapter 3, 4.1.3) a cell appears to be occupying the R_6 position when mitosis is taking place and cells R_1 and R_7 cannot be distinguished. Since the second mitotic wave consists of single division events, then if the equatorial cells are taken up sequentially as suggested, then cells R_6 and R_7 would not be derived from the same terminal division and in this respect should be clonally distinct. However to test this experimentally would be very difficult. Firstly, as has been demonstrated by Lawrence and Green (1979), to be sure that there is no mixing of cells from different clones the frequency of clone production must be very low. Secondly, since it is the terminal divisions that are being investigated then both cells from the mitosis must be labelled. Thirdly, because the frequency of the clones will be low then the receptor cells of a clone

must be readily distinguishable. A mutation such as ora could be used to facilitate identification of labelled receptor cells but a second mutation that can be used to label the other cell of a clone is not located sufficiently close to the ora locus to produce the very low frequency of clone production required.

Poodry et al, (1973) by heat shock pulses to the temperature sensitive mutations shibire during the third larval instar were able to elicit a band of disruption in the adult retina, the anterior positioning of which was dependent upon the age of the subject larva. The anterior displacement with time of the band of disruption correlates strongly with the displacement of the second mitotic wave (see Campos-Ortega and Hofbauer, 1977) and since the second mitotic wave is now known to be associated with the MF then it can be inferred that the target tissue of the heat shock pulses is in the region of the MF.

van Breugel et al, (1975) by injecting third instar larvae with actinomycin D and Fluorouracil (which interfere with RNA transcription and translation respectively) produced a similar band of disruption to that found with shibire. The mutation Notch is known to respond in a similar manner to shibire (Foster and Suzuki, 1970) and van Breugel et al, (1975) have suggested that all disruptions are resulting from interference with differentiation.

For differentiation to be facilitated, RNA synthesis is needed, but the autoradiographical investigation has revealed that RNA synthesis is not increasing in the region of the MF, the label being most densely found in the mature ommatidial bundles particularly in the developing

optic stalk, this being a considerable distance from the MF. However the macrophage-like cells which are regularly found under the basement membrane anterior to the mature bundles are found to show heavy label. The function of these cells is not known but they may be involved in some way in the projection into the optic stalk by the receptor cells, but there is no evidence to support this.

Since RNA synthesis is not noticeably increasing in the region of the MF then the proposal that the induced disruptions are resulting from interference with differentiation must be questioned. Since cellular determination is known to be occurring in the region of the MF then the disruptions may be resulting from interference with this. Since it has been demonstrated that up to the terminal division the retinal cells are not determined (Lawrence and Green, 1979) and that the terminal division is occurring in the MF, then mitosis and the determination are very closely associated. Cells in mitosis are particularly vulnerable to X irradiation and such treatment produces a similar band of disruption to that induced by the other methods mentioned (van Breugel et al, 1975). Fluorouracil is known to interfere with DNA synthesis (Rizki et al, 1972) as is actinomycin D (Mueller and Kajiwara, (1966) - see Monesi, 1972). Perez-Davila and Baker (1967) were able to completely suppress mitosis in Drosophila larvae fed on food containing actinomycin D. It seems more likely, therefore, that disruptions are occurring from interference with the second mitotic wave and the closely associated determination process. It would be interesting to see what the effect of injecting third instar larvae with colchicine would be.

CHAPTER 5. ANALYSIS OF THE MUTATION SEVENLESS

1. Introduction

The studies described so far have dealt with the retinal development of the wild type. The comparison of aberrant with normal development can often provide information of how a developmental process occurs. The third larval instar is an important retinal developmental period in Drosophila and many developmental failures are known to occur from the expression of mutant genes during this stage. Since a more comprehensive understanding of the retinal developmental process occurring during the third larval instar has now been acquired from the earlier work in this thesis, it is expected that failures occurring in retinal determination and differentiation during this period should be more easily recognised and understood. It was decided that an appropriate mutation should be studied, firstly to assess the value of the knowledge that has accrued from the studies of wild type development, and secondly, to derive information of how the particular retinal developmental aberration occurs.

The mutation sevenless was first isolated by Benzer in 1973 and is located on the X chromosome at map position $1-33.2 \pm 0.2$ (Harris et al, 1976). This mutation was chosen for investigation, from a number of suitable mutations, for two major reasons. Firstly, this mutation has been extensively studied (Campos-Ortega et al, 1979; Harris et al, 1976), and secondly, it has a precise and regular effect - the adult ommatidial

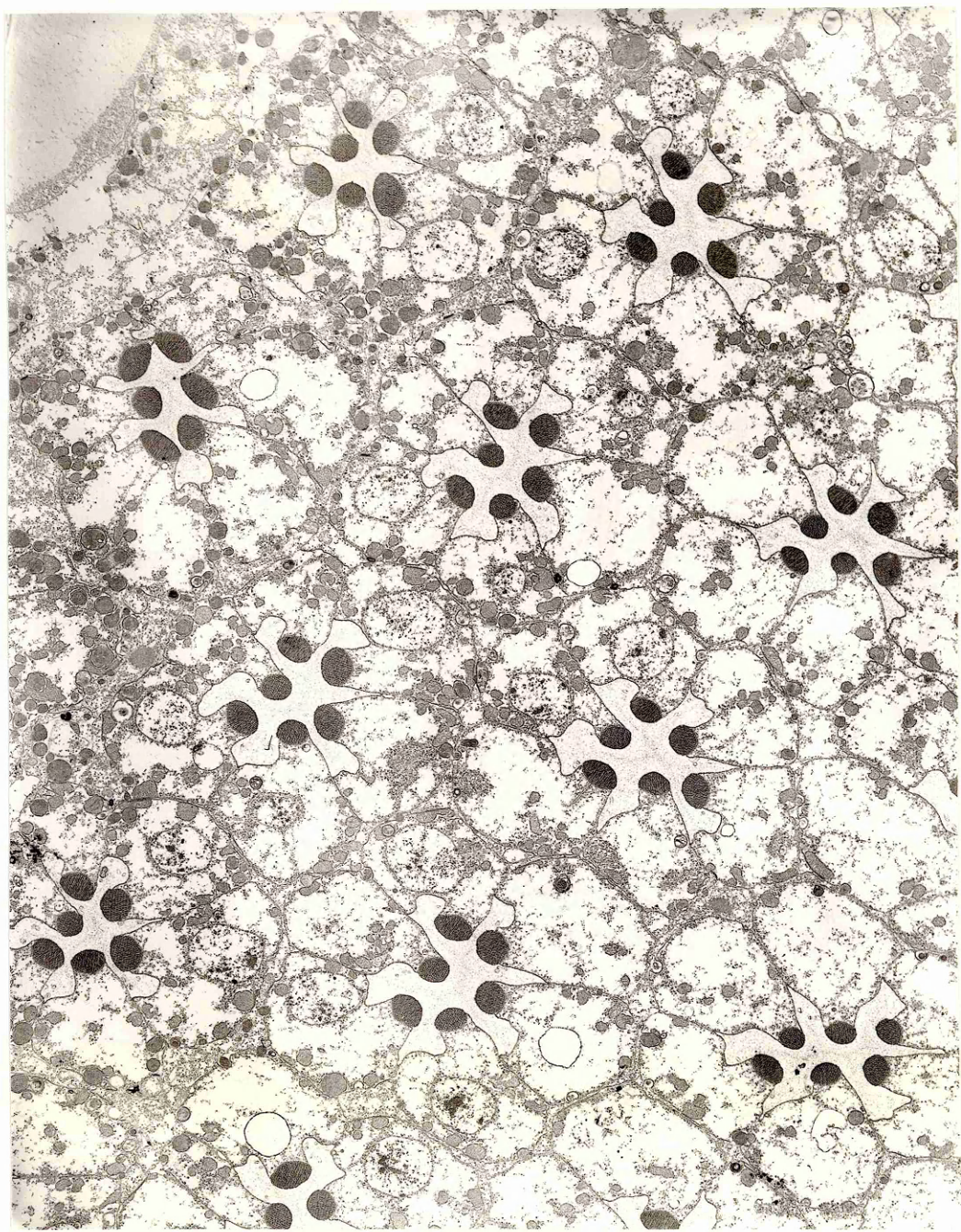


Fig. 5.1a Section through the distal region of the adult retina of sev. Note the presence of only six receptor cells.

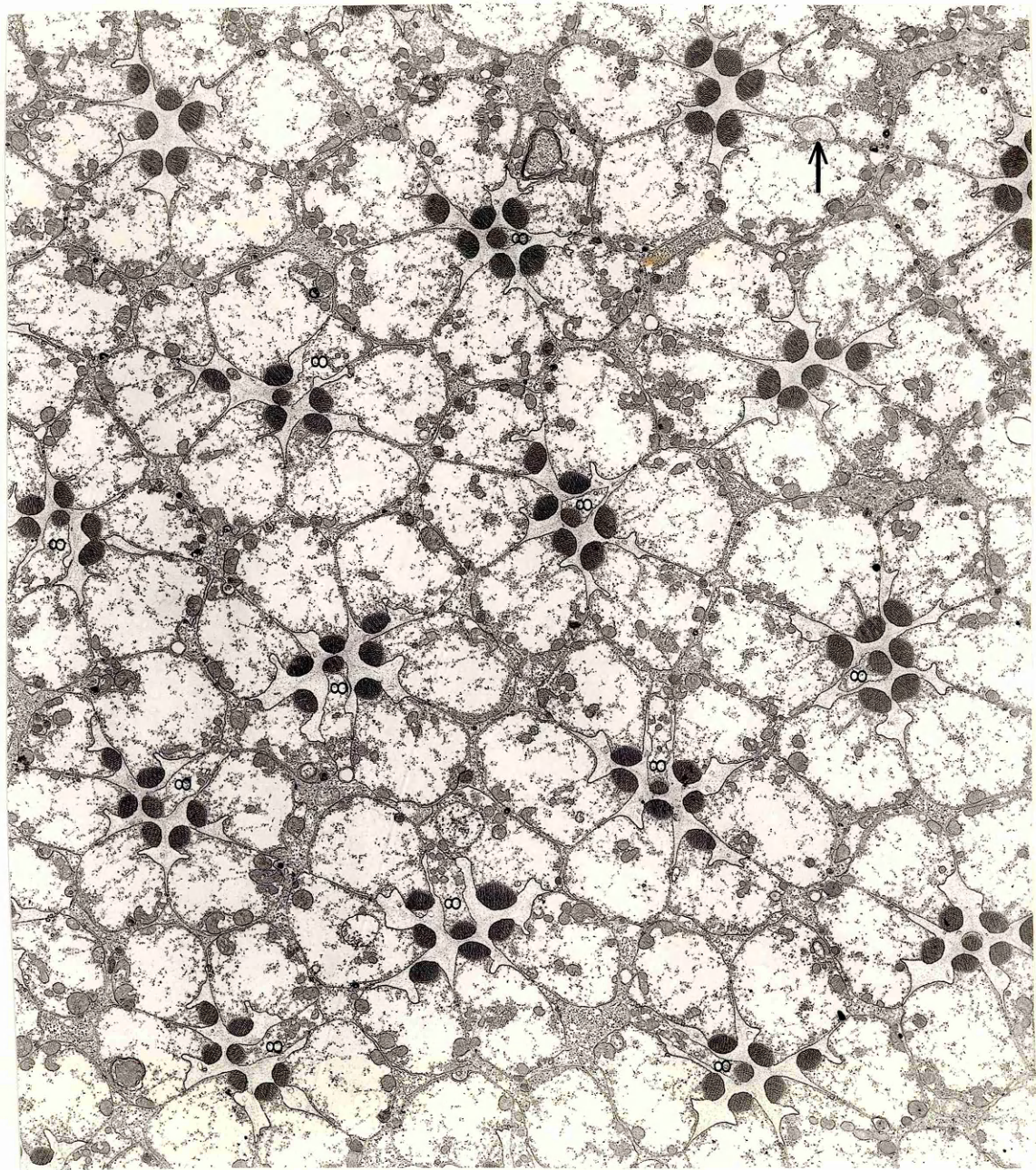


Fig. 5.1b Section through the proximal region of the adult retina of sev. Note the variable positioning of cell R₈, and the remnant of cell R₇ (arrow).

constitution appearing normal except for the apparent lack of the cell R_7 (Fig. 5.1).

Campos-Ortega et al, (1979) examined the third larval instar retinal tissue of sevenless (sev) and only detected mature ommatidial bundles containing seven receptor cells. An analysis of cell death revealed no difference between sev and wild type and they concluded that cell R_7 was not being formed, or possibly not clustering. However, since it is now known that there are a number of mature ommatidial stages, and that what appear to be five cell pre-clusters are in fact eight cell immature clusters, then it was thought that a further investigation of sev third larval instar retinal tissue may well prove fruitful. To this end an electron microscopic investigation was performed upon sev retinal tissue.

1.1 Results

Examination of the posterior region of the MF revealed no obvious differences from the wild type, but since cell identification is more difficult here than in more mature tissue, little importance was attached to this. The unilateral furrowing mechanism was again detected. The tissue posterior to the MF, where the immature ommatidia bundles are found, was now scrutinised. The cellular constitution of this area appeared normal. Immature bundles containing the full complement of eight cells, arranged in their expected positions, were always found. Thus, immature development appeared as in the wild type, cell R_7 being produced and its initial clustering occurring normally.

Aberrant development was not detected until the mature ommatidial stages were examined. Posterior to the immature stages, Stage I mature ommatidial bundles were found. Fig. 5.2 shows sections through one such bundle and it can be seen (Fig. 5.2a) to be displaying the essential cell-body arrangement of a wild type Stage I mature bundle (cf. Fig. 3.6). All receptor cell-bodies are arranged in their expected locations with that of cell R_7 being located most proximally at the equatorial border of the central cell where the other receptor cells are represented by their developing axonal projections (Fig. 5.2b). However, unlike the wild type Stage I bundle, the cell-body of cell R_7 does not bear a developing axonal process proximally. A proximal projection does emanate from cell R_7 but it does not appear as a developing axon and is not included in the axonal bundle which is found to contain only seven elements (Fig. 5.2c). Many Stage I bundles were examined and all were found to display the characteristics described. However, a tendency was detected, at differing levels between the cell-body of R_7 and the lumen surface, for the cell R_7 to be excluded from central cell contact, it being occluded by cells R_1 and R_6 . Even when this was occurring, the cell-body of R_7 was consistently found in the correct Stage I location bordering the central cell. Thus, upto and including the Stage I ommatidial bundle, the cell R_7 appears to cluster normally but fails to undergo neurogenesis and hence does not adopt a receptor cell developmental pathway.

Examination of the tissue to the rear of the retinal region revealed that during later third larval instar development, cell R_7 loses its clustering position and the ommatidial bundles are of distinctly aberrant appearance. Fig. 5.3 shows sections through a more mature

ommatidial bundle and it can be seen in a section of tissue close to the distal surface that cell R_7 has now risen up the clustering (Fig. 5.3a). The presence of the two cone cells clustered distally indicates that this is a Stage II mature ommatidial bundle, but the receptor cell arrangement is not as found in the wild type. Fig. 3.5a shows the typical distal appearance of the receptor cells and it can be seen that cell R_7 is precluded from central cell contact. However, the positioning of cell R_7 , although normally found as in Fig. 5.3a, was found to be somewhat variable - sometimes persisting in central cell contact and hence appearing as wild-type, and other times being excluded from eccentric receptor cell contact by the two cone cells (Fig. 5.3b).

In the proximal regions of the clustering, cell R_7 is always found to regain contact with the central cell, this being particularly evident at levels just above the position where the axonal bundle begins. Here cells R_1 and R_6 now allow R_7 to gain contact with the central cell (Fig. 5.3d) but as in the earlier stages, cell R_7 fails to project a developing axon and axonal bundles containing only seven elements project into the developing optic stalk (Fig. 5.3e).

In this more mature ommatidial bundle, particularly in the proximal regions of the cell-body clustering, the cytoplasm of the receptor cells is noticeably less dense than the surrounding tissue, this not being apparent in the wild type. Examination of cell R_7 in the more mature bundles has consistently revealed it to appear as a non-receptor cell.

The Stage III mature ommatidial bundles were not examined.

FIGURE 5.2

Sections taken from a Stage I mature ommatidial bundle of sevenless. All receptor cells are numbered.

Fig. 5.2a x 10,000. A section through the distal clustering, note the essential Stage I appearance.

Fig. 5.2b x 10,000. The nucleus of R₇ is the most proximally placed and it can be seen that the cell occupies its wild type positioning.

Fig. 5.2c x 10,000. Only seven elements make up the axonal bundle which projects into the optic stalk.

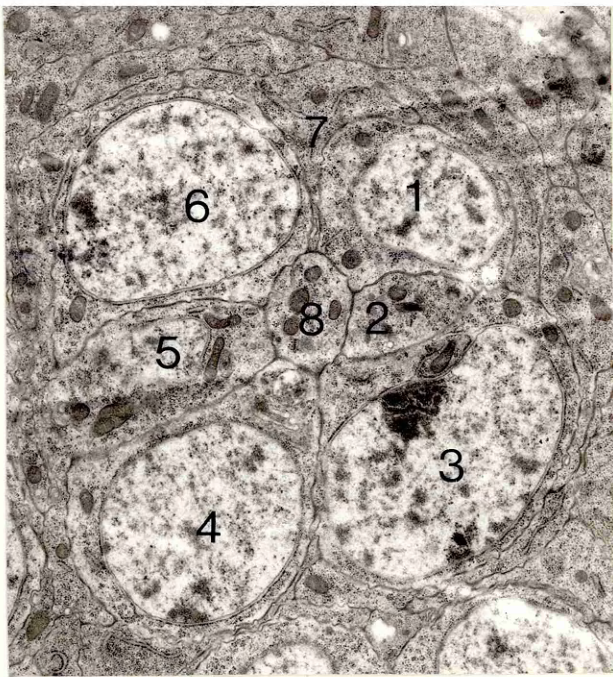


Fig. 5.2a

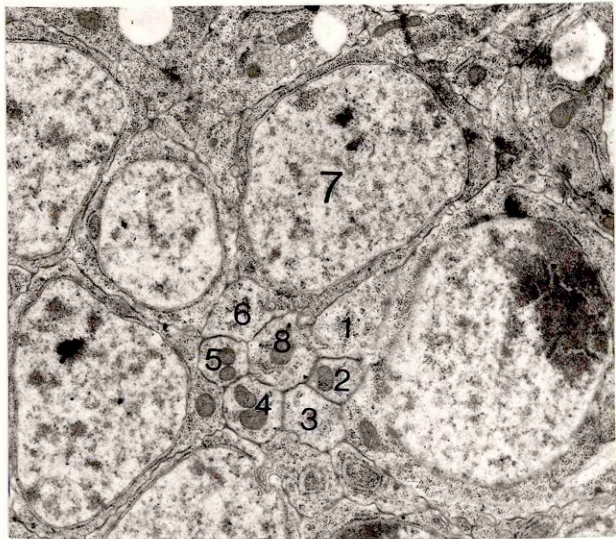


Fig. 5.2b

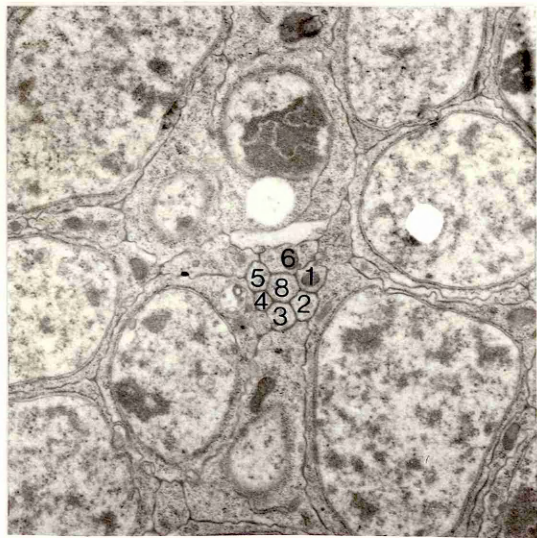


Fig. 5.2c

FIGURE 5.3

Sections taken from a Stage II mature ommatidial bundle of sevenless. All receptor cells are numbered. All sections except Fig. 5.3b are from the same bundle.

Fig. 5.3a x 10,000. The typical appearance of the distal clustering. Note the presence of the two cone cell nuclei (C). R_7 can be seen occluded from its normal contact with R_8 .

Fig. 5.3b x 10,000. In the distal clustering the two cone cells (C) can occlude R_7 from receptor cell contact.

Fig. 5.3c x 10,000. Section through the lower cell-body clustering. Note the distinct appearance of the receptor cells and the rising cone cell (C).

Fig. 5.3d x 10,000. At levels just above the beginning of the axonal bundle, R_7 regains contact with R_8 .

Fig. 5.3d x 10,000. Only seven axons project into the optic stalk.

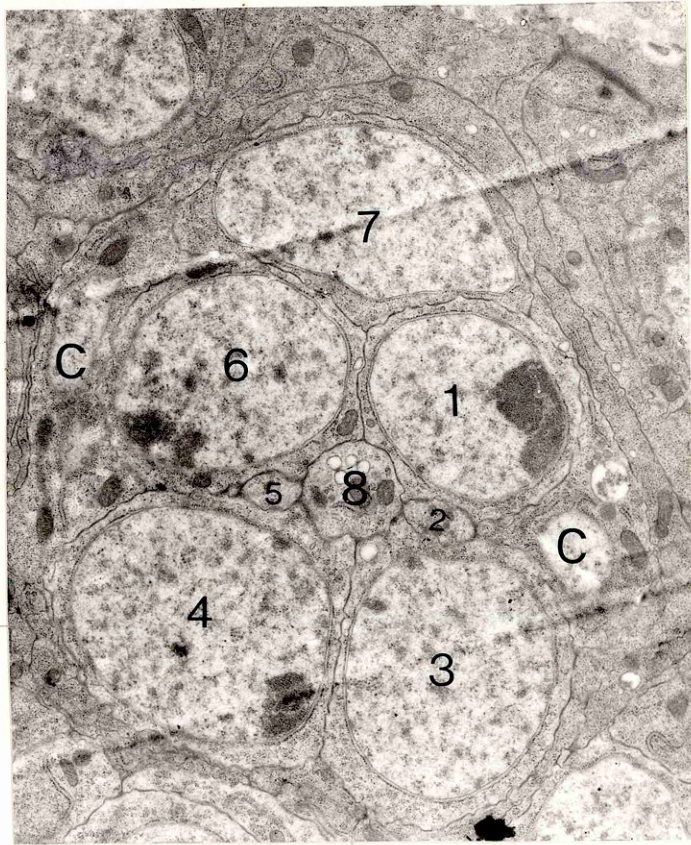


Fig. 5.3a

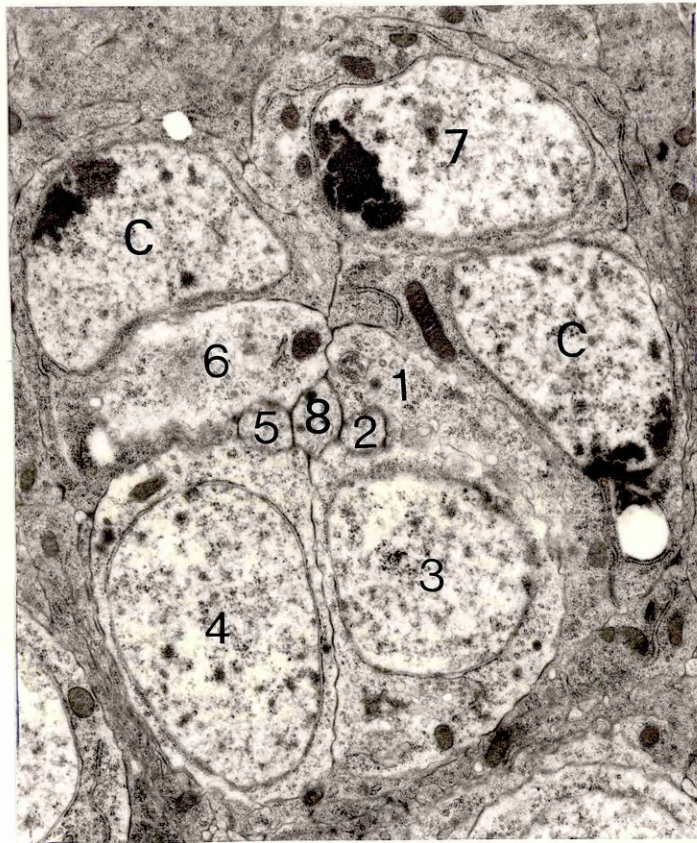


Fig. 5.3b

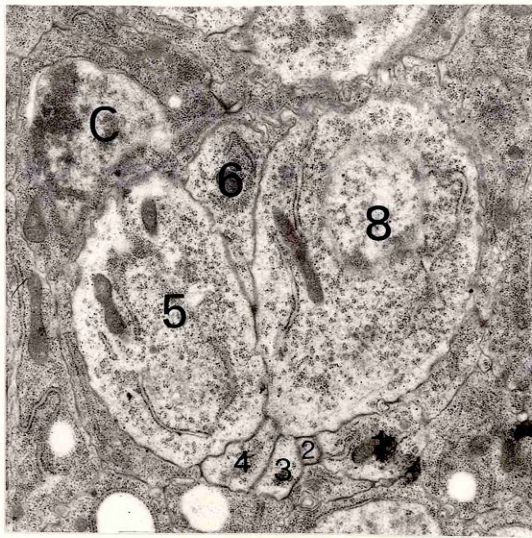


Fig. 5.3c



Fig. 5.3d

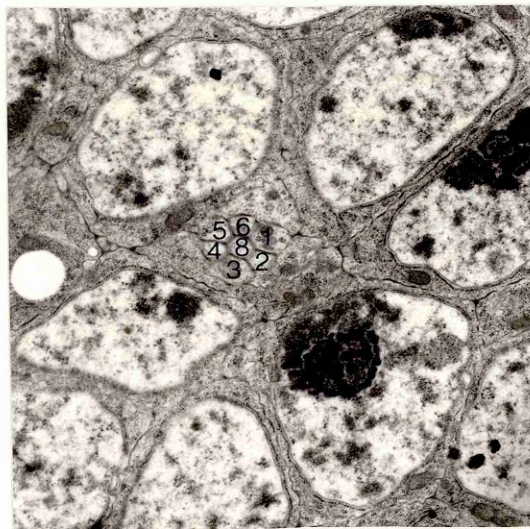


Fig. 5.3e

2. DISCUSSION

2.1 The presence of cell R₇

Campos-Ortega et al, (1979) performed a detailed investigation of the development of the mutation sev and produced many important findings, but failed to recognise the presence of the cell R₇ in the developing ommatidial bundles. It is now known that this cell is produced, initially clusters correctly, and only in the more mature ommatidial bundles does it lose its clustering position. It is possible that Campos-Ortega et al, (1979), assuming the immature bundles to be pre-clusters, examined the tissue at the rear of the retinal field to ensure that mature ommatidial bundles were investigated. If this was the case then it would have been very easy for them to overlook the Stage I tissue and in the more mature stages, where cell R₇ is somewhat displaced from the clustering, this cell could have been easily confused with a cone cell.

Campos-Ortega et al, (1979) described the adult retina of sev as completely lacking cell R₇, however, Harris et al, (1976) found the small undifferentiated cell-body of R₇ to be present. Examination of the adult retina by the present author has confirmed the presence of a small cell-body (Fig. 5.1b), usually found in the distal half of the retina, but a detailed investigation to establish whether it is present in all ommatidia was not carried out. It seems likely therefore than in the developing sev retina, the cell, which in the wild type becomes R₇, degenerates during the pupal stage.

2.2 The cause of the sev phenotype

In the more mature bundlings the receptor cells are cytoplasmically distinguishable from the non-receptor cells, which was not thought to be the case in the wild type . It should be stressed that the stock used was w sev, but the white gene would not account for this unless some pleiotropic effect was operating. However, close scrutiny of the most mature tissue in the wild type reveals that the cytoplasm in the receptor cells is slightly less dense than the other cells - this becoming very apparent in the adult retina. Knowing that cell R_7 is the last receptor cell to differentiate then it might be thought that this cell is failing to differentiate as a result of not receiving the correct developmental cues from its precociously differentiating receptor cell counterparts. But, Campos-Ortega et al, (1979) by clonal analysis have shown that sev⁺ R_7 cells are found in otherwise sev ommatidia and hence it can be concluded that in the sev phenotype the cell R_7 is not prevented from developing correctly by the other cells, the presence of the sev genotype in the cell R_7 , alone being responsible.

The question now arises as to why the cell which initially clusters correctly then fails to undergo differentiation into R_7 . Either the developmental failure occurs prior to, or after, cluster formation. If the developmental failure is prior to cluster formation then this implies that in the wild type cell R_7 is determined prior to entering its clustering position and that it is generated by a receptor cell mother cell. Although cell lineage has been largely ruled out in later retinal development by the clonal analyses hitherto described, the terminal division itself cannot be assessed and hence cells produced

by the second mitotic wave may, to some extent, be derived from specific cells i.e. equatorial receptor cells being generated by receptor cell mother cells. However, this seems unlikely. The indication from Lawrence and Green (1979) is that the second mitotic wave consists of single division events and if receptor cell determination is established prior to the terminal division then four receptor cells will be produced equatorially to half of the pre-clusters one of which must migrate to an adjacent grouping. This seems unlikely since the immature bundles form immediately after the second mitotic wave and little time exists for a cell to undergo the appreciable migration necessary. It still cannot be completely ruled out however that the equatorial receptor cells of each ommatidium are produced from a double division generating three cells. But if this is the case it is difficult to understand how in sev, a non-receptor cell could come to occupy the R_7 position. Since R_1 and R_6 are produced then the third receptor cell would be expected i.e. a division failure of a receptor cell mother cell would produce ommatidia lacking two or three of the equatorial receptor cells not one. Hence on the above arguments the theory that a non-receptor cell clusters in the R_7 position can be discarded.

This now leads to the second possibility that the cell destined to be R_7 does not become committed to this development until after it has entered the cluster. In sev, the cell R_7 initially clusters correctly but then does not only fail to undergo the R_7 developmental pathway, but it also fails to undergo general receptor cell development. The sev locus is known to act specifically on the cell R_7 (Campos-Ortega et al, 1979) and hence it can be concluded that general, and specific

receptor cell development are, in this particular cell at least, intimately linked. The sev locus itself need not necessarily be defining cell R_7 , it may be one of a number of loci involved in this process or the sev phenotype may result from a secondary effect of the locus being occupied by the mutant allele, this affecting the epigenetic pathway which controls the development of the cell R_7 .

2.3 The effect of the lack of cell R_7 on retinal development

In the wild type adult retina cell R_8 is located between cells R_1 and R_2 , but in the adult retina of sev this is not necessarily the case. Fig. 5.1b shows the ommatidia at levels where cell R_8 is present and it can be seen that it is not regularly positioned as in the wild type, but is found in a variety of locations.

One of the cues used in cell identification in the developing ommatidial bundles is the cell R_7 itself, this cell providing structural polarity to the clustering. It is possible that cells in the developing ommatidial bundles use cell R_7 to establish the polarity of the clustering, and hence in sev since the cell is displaced from the bundlings the receptor cells are failing to organise properly. The receptor cells are distinguishable into three groups - R_7 , R_8 , and R_{1-6} (see Harris et al, 1976) and these groupings may already be established in the third larval instar. Thus the central cell (R_8) would find itself surrounded by identical cells and would not be able to distinguish cells R_1 and R_2 .

Ready et al, (1976) have described the retina of Drosophila as a neurocrystalline lattice. This chemical analogy is confusing, it is not made clear whether the individual ommatidium or the entire retina is regarded as the unit crystal. The variable positioning of cell R_8 in the sev retina indicates that retinal assembly occurs in the unit ommatidium, cells not being recruited onto an advancing organised cellular front. If the latter was the case then polarity would already be established in the organised front and cell R_8 would be regularly positioned between cells R_1 and R_2 .

There is further evidence that the individual ommatidium is self assembling. Experiments have been reported where treatments for a limited period during the third larval instar induce a band of disruption in the adult retina (Poodry et al, 1973; van Breugel et al, 1975). If retinal cells were recruited onto an advancing front of cellular organisation then the disruption would be expected to spread to the anterior of the eye. The disruption of the front of the organisation would prevent the correct uptake of cells onto the front and thus the new front itself would be disrupted. Hence, the retinal cells can be viewed as assembling themselves into the ommatidial structure as they emerge from the morpho-genetic furrow.

This again challenges the idea of the differentiation front traversing the retinal field during the third larval instar. The anterior spread of the differentiated tissue during the third larval instar results from the temporal delay in retinal tissue generation over the antero-posterior axis of the eye field.

CHAPTER SIX : Conclusions

The processes now known to occur in the retinal tissue of the third larval instar are here briefly summarised.

During third larval instar retinal development, ommatidial assembly is first initiated posteriorly and the ommatidial region extends anteriorly during the course of the instar. Because the wave of ommatidial production progresses anteriorly then the antero-posterior axis during this period can be considered as one of time, the series of developmental stages evident along this axis are those through which all the retinal cells pass (Fig. 6.1).

The retinal field is initially proliferated by the anterior mitotic wave. The nature of the proliferative wave remains unclear, it still needs to be established whether it is an advancing eye margin or a mitotic wave traversing a presumptive retinal field. Shortly after generation by the anterior mitotic wave the cells contract in the proximo-distal axis, this being manifested as the morphogenetic furrow (MF), and the second mitotic wave is now initiated, the division occurring in the posterior aspect of the MF as the cellular contraction is being reversed. The immature ommatidial bundles are generated by the second mitotic wave and immature development leads to the mature ommatidial constitution where all receptor cells have projected into the developing optic stalk. Mature development consists of an orderly rise of the cone cells from proximal positions with concomitant receptor cell rearrangement establishing the essential adult constitution

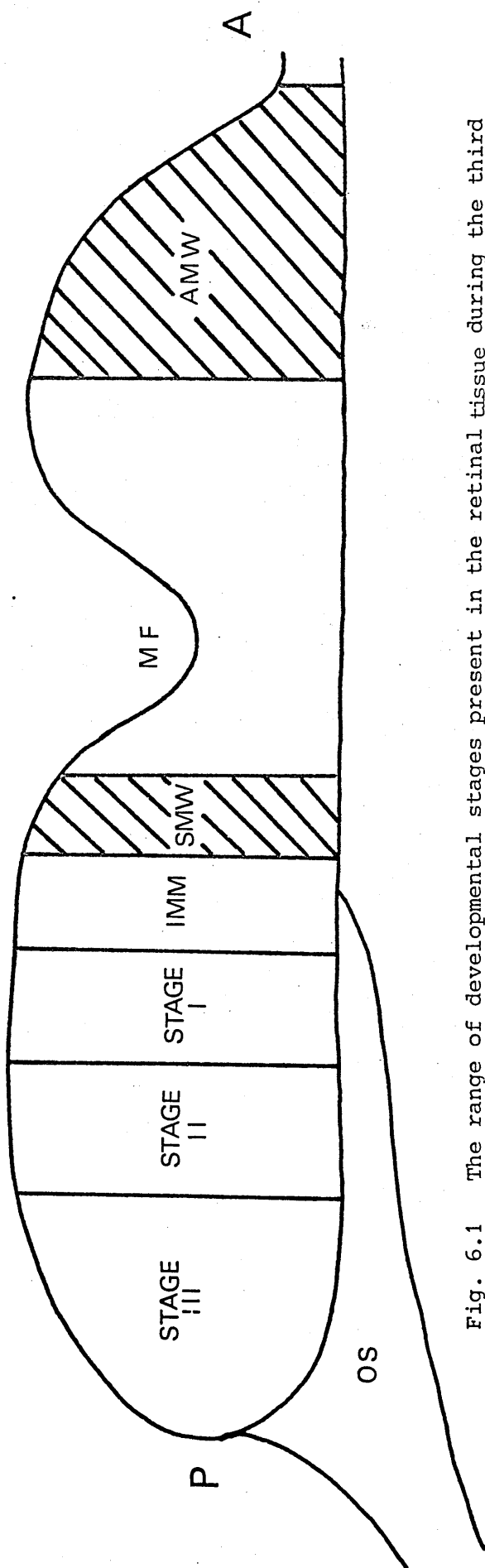


Fig. 6.1 The range of developmental stages present in the retinal tissue during the third larval instar. AMW: anterior mitotic wave; MF: morphogenetic furrow; SMW: second mitotic wave; IMM: immature development; OS: optic stalk; A: anterior; P: posterior.

of the distally placed dioptric cellular system overlying the receptor cell bundlings.

Analysis of the mutation sevenless (sev) has not only revealed the value of the knowledge acquired from examination of the wild type but has also explained the cause of the sev phenotype. The missing cell, hitherto thought either not to be present or cluster, is now known to initially cluster correctly, fail to undergo a receptor cell developmental pathway, become displaced within the cluster and eventually degenerate.

APPENDIX

Three Dimensional Reconstruction of the Retinal Tissue using Computer Graphics

Introduction

Data derived from serial sectioning is often difficult to envisage in its true three dimensional appearance without some form of physical representation being used. Physically constructed models or hand drawings were not considered sufficiently suitable for the rather complex cellular organisation occurring in the imaginal retinal tissue of Drosophila. It was decided that since a system of computer programs were being developed in this department for reconstructing serially sectioned material that this should be used.

Use of the programs

The outline of the relevant cells from each section were first transferred to tracing paper and a Tektronix 4663 Digitizer was used to input the data into a DEC VAX 11/780 computer. Twenty four points per cell were digitised, each point being recorded as the X and Y displacements from a central fixed origin.

Using an 80 x 80 integer array the cellular outlines could be displayed, for each cell the points lying between two sequentially digitised points being interpolated. The image of the stacked sections could then be viewed.

The reconstructed ommatidial bundle could be rotated in the X/Y plane and the angle of inclination of observance could also be adjusted, the latter remaining constant for the print-outs presented in this thesis. A hidden lines program routine prevented those parts of the ommatidial bundle that should be obscured from view when observed from any particular position from being drawn.

The cells of the ommatidial bundles are intimately associated, and to aid visual appreciation of the structure a program was utilised which allowed the cells to be slightly separated from each other.

Rotation of the image of the ommatidial bundle gave its overall external appearance, but use of a program feature allowing any number of individual cells to be removed enabled a more thorough understanding of the entire constitution of the bundle to be acquired.

Although each individual cell could be displayed in a separate colour it was found for the ommatidial bundles that a monochrome display using broken and continuous lines was perfectly adequate and, in terms of print-out, considerably less expensive.

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